The Journal of Laboratory and Clinical Medicine

VOL. 28 SEPTEMBER, 1943 NO. 12

CLINICAL AND EXPERIMENTAL

BLOOD PRESSURE FLUCTUATIONS IN BRONCHIAL ASTHMA*

II. EFFECT OF EPINEPHRINE AND AMINOPHYLLIN

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THE occurrence in bronchial asthma of excessive fluctuations of the systemic systolic blood pressure, synchronous with the respiratory cycle, has been noted in the past by a number of observers, among whom are Tinel and Jacquelin,[1] Rackemann,[2] and Feinberg.[3]

In a recent article[4] one of us drew attention to these fluctuations, and reported observations on a number of asthmatic patients under various circumstances. The conclusions, which agreed closely with those of Tinel and Jacquelin, were: (1) During the asthmatic paroxysm there is constantly present an increase above normal in the respiratory fluctuation of the systolic blood pressure. (2) The amplitude of this fluctuation parallels closely the severity of the asthmatic dyspnea. (3) When the asthmatic paroxysm has ended, the respiratory systolic fluctuation returns to normal limits. An exception to the third statement has been noted: In certain chronic asthmatic patients, in whom irreversible changes have taken place in the lungs, there may be present at times a moderate increase in the respiratory systolic fluctuation, even though the patient appears to be in little or no distress.

Further observations disclosed that the systolic blood pressure uniformly reaches the highest point during expiration and falls to its lowest point during inspiration, both during the paroxysm and during the asthma-free state. Certain experiments with respiratory obstruction in anesthetized cats and in healthy young men, which Osgood has reported,[5] showed a similar increase in the respiratory systolic fluctuation with increasing obstruction. The timing of the high

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Received for publication, December 23, 1942.

and low points of the fluctuation in the respiratory cycle was also the same in these experiments as in bronchial asthma.

The detection and measurement of this respiratory systolic fluctuation are readily made, using an ordinary sphygmomanometer. The method was described in a previous paper.[4] The normal fluctuation in healthy persons with quiet breathing averages about 4 mm. Hg with a range from 2 mm. to 8 mm.

The present paper concerns the effect on this fluctuation of epinephrine and of aminophyllin, administered for the relief of bronchial asthma.

Relief from the dyspnea of bronchial asthma induced by the subcutaneous injection of epinephrine is usually prompt in appearance. It is evidenced subjectively by a rapid diminution of the respiratory distress, and objectively by a decrease or disappearance of the asthmatic wheezes and râles from the lungs; or, if there is much mucus present, by a coarsening of the asthmatic sounds in the chest, and sometimes by the expectoration of secretion. There is also a lessening or a complete cessation of the vigorous muscular efforts of respiration and of the retraction of the suprasternal and supraclavicular tissues.

Following the intravenous administration of aminophyllin with relief, the changes are somewhat different. They are not so marked and are often slower in appearing. They consist subjectively of a feeling of general relaxation and of release from dyspnea, which may be prompt or delayed one-half to one hour, and sometimes of a fleeting feeling of warmth. There may also be a cough with evacuation of sputum. Objectively there is a decrease in the respiratory efforts, as with epinephrine. The retraction of the soft tissues of the chest and the asthmatic sounds in the lungs, however, are apt to diminish to a lesser degree than the subjective relief would seem to indicate. After both drugs it is not difficult to estimate clinically the degree of relief afforded, utilizing these various phenomena.

BLOOD PRESSURE FINDINGS AFTER EPINEPHRINE

Charts I and II present the blood pressure findings in seven patients, all suffering from moderate or severe bronchial asthma at the time. As on all subsequent charts, the high and low points reached by the systolic blood pressure during expiration and inspiration, respectively, are shown in mm. Hg, and the respiratory fluctuation is indicated by the cross-hatching between these points. The degree of dyspnea at the moment of each reading is indicated by the solid black column at the bottom. Below the column is marked the time of the injection of medication and of each reading.

It will be noted that the systolic blood pressure showed wide fluctuations before the epinephrine was given. Within ten to twenty minutes after the injection the systolic fluctuation had returned to within normal limits in those patients who obtained clinical relief.

Patient F. N. (Chart I), suffering from chronic asthma of the "mixed" type, secured prompt relief, and in twenty minutes following the injection of epinephrine the systolic fluctuation had dropped from 14 mm. to 3 mm. On the other hand, M. P. (Chart II), whose chronic asthma was also of the mixed type, obtained practically no relief, and the respiratory systolic fluctuation, which had been 28 mm. beforehand, was still 23 mm. thirty-three minutes after the injection.

The patient C. B. (Chart II) suffered from chronic asthma of the intrinsic type associated with hyperplastic sinusitis. His severe, recurrent attacks were usually relieved temporarily by epinephrine. A reading in May, 1940, during a severe paroxysm, showed a systolic fluctuation with respiration of 24 mm. On August 3, 1940, he was seen in great distress, with his skin gray and sweating. The respiratory systolic fluctuation was 50 mm., one of the greatest we have observed. He was given ½ c.c. of 1 to 1000 epinephrine and 1 c.c. of epinephrine in oil, 1 to 500. He experienced marked relief, and twenty minutes after the injection the systolic fluctuation had dropped to 6 mm. A reading eight months later, when he was in no apparent distress, gave a systolic fluctuation of 12 mm. His lungs, however, showed numerous coarse wheezes throughout.

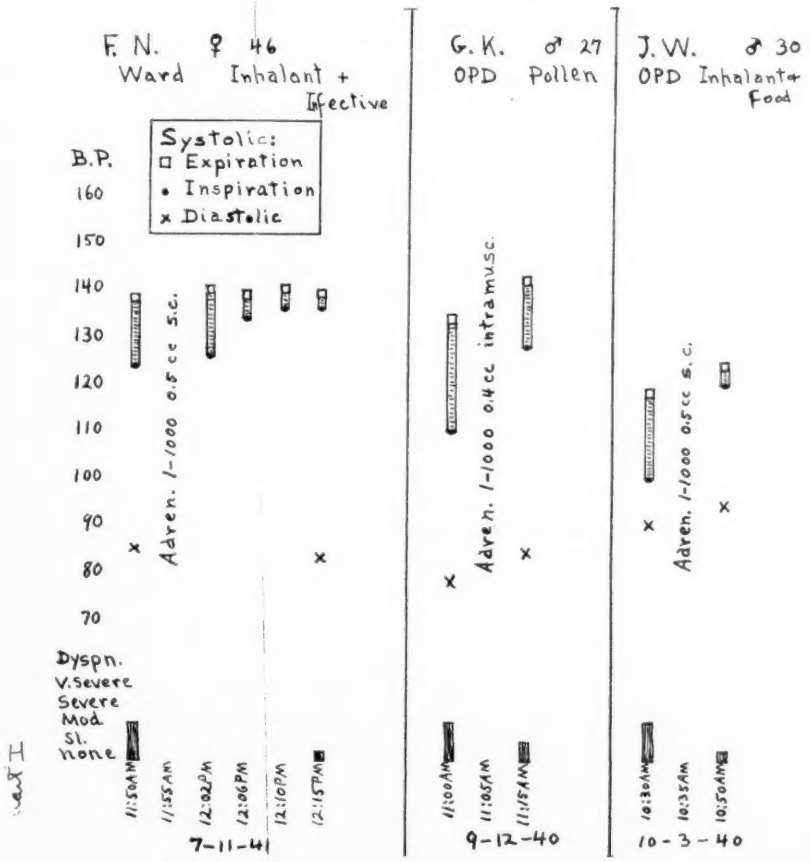

Chart I.—The effect on the respiratory systolic fluctuation of epinephrine administered for the relief of bronchial asthma.

BLOOD PRESSURE FINDINGS AFTER AMINOPHYLLIN

The response of the respiratory systolic fluctuation to the intravenous injection of aminophyllin differs from that of epinephrine. On Charts III and IV are recorded observations on two patients to whom epinephrine and aminophyllin were given at different times for relief of their asthma.

Patient A. B. (Chart III) was sensitive to tree, grass, and weed pollens, and to some other unidentified factors, probably intrinsic. Her paroxysms were periodic and severe, and epinephrine usually gave relief. On April 17th she was seen in a severe attack; her lungs showed many wheezes. The respiratory fluctuation of her systolic pressure was 24 mm. seated, and 38 mm. recumbent. Six-tenths c.c. of 1 to 1000 epinephrine was given subcutaneously, while recumbent. Sixteen minutes later she had no distress and the respiratory systolic fluctuation was 6 mm. recumbent. A week later she was again seen in a moderately severe paroxysm with wheezes throughout her lungs. The respiratory systolic fluctuation was 26 mm. Aminophyllin, 0.24 Gm. in 10 c.c., was given intravenously. Twenty-five minutes later she had experienced considerable subjective relief, although not complete, and her lungs still showed many wheezes. The respiratory systolic fluctuation was still 24 mm.; the expiratory peak of her systolic pressure had fallen from 120 mm. to 110 mm.

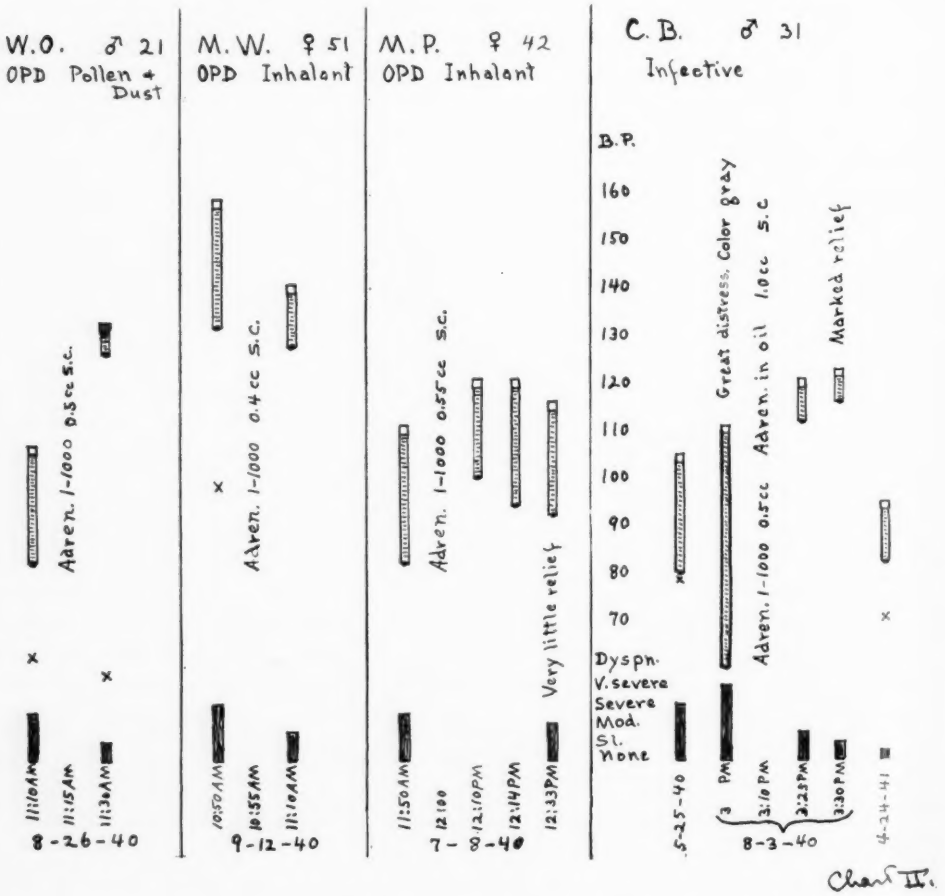

Chart II.—The effect of epinephrine on the respiratory systolic fluctuation in bronchial asthma.

Patient G. H. (Chart IV) was exceedingly sensitive to grass and weed pollens and possibly to molds; her asthma was seasonal. She was seen on July 7 when suffering from severe asthma. The respiratory systolic fluctuation was 18

mm. Epinephrine, 0.8 c.c. in 1 to 1000 solution, was given subcutaneously. Subjective relief was felt in eight minutes, and was marked, though incomplete, in twelve minutes, at which time the respiratory systolic fluctuation was 8 mm. On September 6 she was again having severe asthma. One-half c.c. of 1 to 1000 epinephrine had been injected by her son at 6 A.M. At 7:30 A.M. she was suffering severely, with "tight" wheezes throughout the lungs. The expiratory peak of her systolic pressure was 180 mm. and the respiratory fluctuation was 15 mm. One-half gram of aminophyllin in 20 c.c. was given intravenously. Ten minutes later the expiratory peak of her systolic pressure had fallen to 96 mm.; fifteen minutes after the injection it was 110 mm. She had great subjective relief and began raising considerable sputum. The respiratory systolic fluctuation, however, was still wide: 16 mm. and 20 mm. on two close readings. Twenty-three minutes after the injection, relief was maintained, but the respiratory swing was still 20 mm. Two and a quarter hours later she was somewhat dyspneic with continued expectoration; the respiratory fluctuation was 14 mm. She required no further medication through the day, but was given 1 c.c. of epinephrine in oil at 7:30 P.M. At 10 P.M. she had slight dyspnea and her expiratory systolic peak was 120 mm. with a respiratory fluctuation of 8 mm. The following morning she was asthma-free. The respiratory systolic fluctuation was 8 mm.

Chart V presents the findings in C. G., a married woman aged 29 years, sensitive to pollens and to a number of other inhalants. On October 19 she had been given adrenalin in oil, 1 to 500, at 4 A.M. and at 11 A.M. At 12:30 P.M. she was markedly dyspneic and orthopneic, with retraction of the soft tissues of the chest and sonorous sounds throughout the lungs. The respiratory systolic fluctuation was 40 mm., with the expiratory peak at 140 mm. She was given 0.25 Gm. aminophyllin in 10 c.c. intravenously. Fifteen minutes later she had felt some relief, but the systolic fluctuation was still 40 mm. Relief was progressive, and two hours and forty-five minutes after aminophyllin she was very comfortable, with only slight dyspnea and some wheezes in her lungs. The respiratory fluctuation of her blood pressure, however, was still 26 mm. Two days later she was free of dyspnea and the respiratory fluctuation was 11 mm. and 14 mm. The lungs still contained some sonorous râles.

J. G. (Chart VI), a colored man of 42 years, had long bouts of severe asthma due to intrinsic factors and to foods. He showed at times frequent premature ventricular systoles, although there were no significant electrocardiographic changes or other signs of cardiac disease. His blood Wassermann was negative. Epinephrine afforded so little relief that he refused its administration and asked for aminophyllin. Before injection of the latter drug he would be sitting up, feet over the bedside, wheezing, coughing, and sweating. After its injection he would quiet down in a few minutes and lie semirecumbent, although rarely did his chest clear of wheezes. As will be noted in the chart, the response of the systolic fluctuation to the drug was rather irregular. In general there was a fall in the level of the expiratory peak and a decrease in the respiratory fluctuation, although not so marked as after epinephrine in other patients.

Chart VII gives the blood pressure readings on patient M. M. taken before and after six intravenous injections of aminophyllin and two subcutaneous injections of epinephrine.

She was an unmarried woman, aged 24 years, sensitive to grass and weed pollens, to orris, and to some other inhalants. She had had recurrent attacks of asthma since childhood, which in recent years occurred chiefly in the summer and fall, and lasted several weeks at a time. She had developed surprisingly few permanent changes in the lungs or chest cage. Three years of hyposensitizing treatment had not produced very satisfactory results. Her paroxysms were difficult to control and she was rather "sensitive" to epinephrine. Best relief was secured with aminophyllin, although it was rarely complete and was slow in appearing.

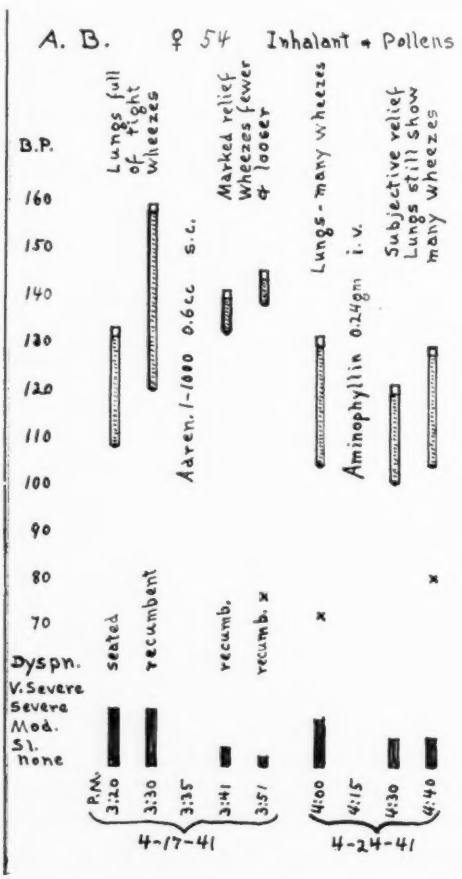

Chart III.—Comparative effects on the respiratory systolic fluctuation of epinephrine and of aminophyllin, administered to a patient in separate paroxysms of asthma.

In her chart it will be noted that with severe dyspnea, the respiratory fluctuation of her systolic blood pressure was wide, varying between 22 mm. and 40 mm. before either drug had been given. After the two epinephrine injections (July 3, 1939, and June 13, 1940) she obtained little or no relief, and the respiratory systolic fluctuation did not decrease in amplitude; the expiratory peak rose slightly.

The six injections of aminophyllin afforded considerable though incomplete relief. She felt "more relaxed" afterwards. Following three of the injections,

the respiratory systolic fluctuation decreased slightly (by 10 mm., 16 mm., and 14 mm. respectively), but did not return even to near normal limits during the period of observation. After the other three injections there was a slight decrease in the fluctuations (6 mm., zero, and 2 mm.). With aminophyllin, such relief as occurred seemed to follow more a moderate fall of the expiratory peak of the systolic pressure than a decrease in the respiratory fluctuation.

Two months later, after she had been asthma-free for five weeks, the respiratory systolic fluctuation was 8 mm.

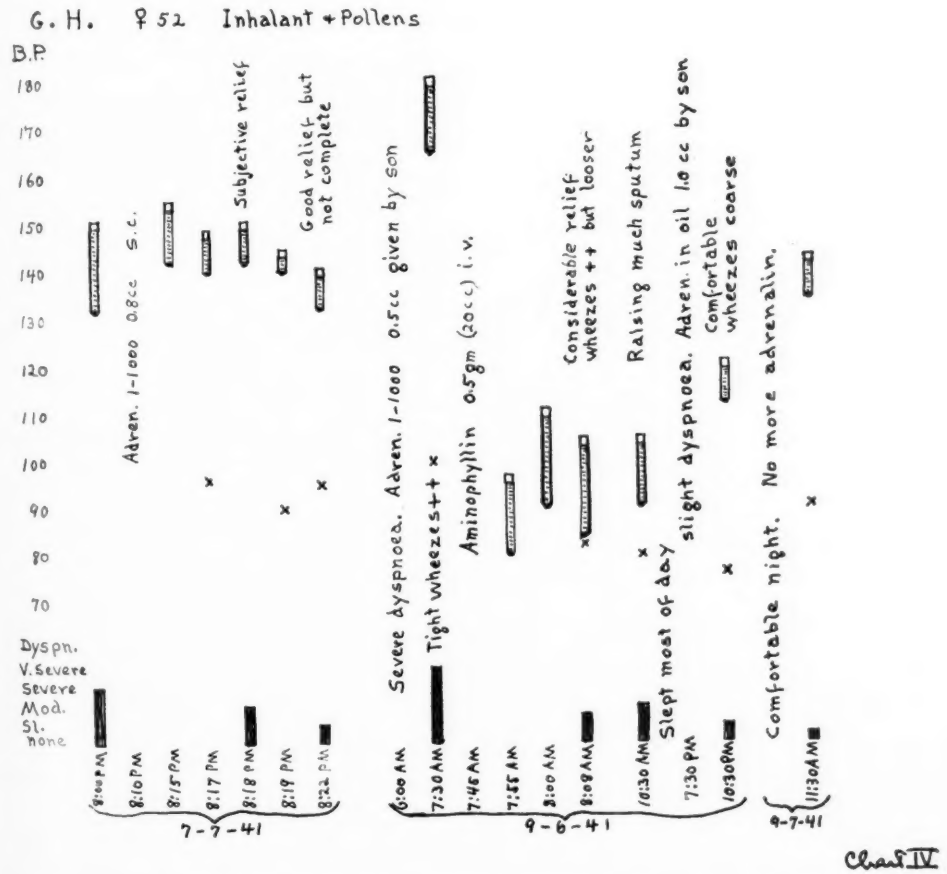

Chart IV.—Comparative effects on the respiratory systolic fluctuation of epinephrine and of aminophyllin, administered to a patient in separate paroxysms of asthma.

DISCUSSION

As a result of observations reported by Osgood in two previous articles,[4, 5] he believes that the enhanced respiratory fluctuation of the systolic blood pressure found in bronchial asthma is dependent upon and is an indirect measure of the obstruction to respiration and that a return of this fluctuation to normal signals a reduction of the obstruction. Consequently the observed return of this fluctuation to normal, when relief follows epinephrine, would indicate that the relief with this drug is brought about by a direct release either of bronchial

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spasm, or of bronchial edema, or of both. When relief is obtained with aminophyllin, judging from the fact that the respiratory systolic fluctuation decreases but does not return entirely to normal limits, we may conclude that there has been clinical evidence of *some* bronchodilatation with this drug also. That the fluctuation does not decrease appreciably, either after epinephrine or aminophyllin, when no relief is secured, indicates that the bronchial obstruction has not been released.

Since relief with aminophyllin does not appear to depend wholly on bronchodilatation, the question arises as to what mechanism is here involved.

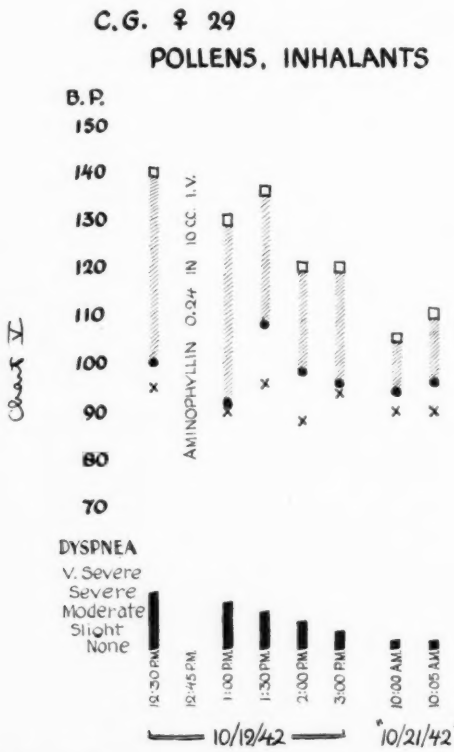

Chart V.—The effect of aminophyllin on the respiratory systolic fluctuation when relief is secured.

Those patients who were given aminophyllin were in general suffering from more obstinate asthma than those given epinephrine, and many were "epinephrine-fast." It is possible that in some of these patients retained bronchial secretion tended to maintain partial obstruction, thus continuing the wide respiratory fluctuation of the systolic blood pressure. This would not, however, explain the rather uniform persistence of a wide fluctuation after aminophyllin with relief.

If the asthmatic patient continued normal respiratory movements during a paroxysm of bronchial obstruction, there would be insufficient ventilation to eliminate properly CO_2 or to take in O_2; hypercapnia and anoxia would result. Leaving out of consideration the possibility that nerve impulses from the lungs, initiated by the respiratory obstruction itself, induce dyspnea, it can be assumed that the asthmatic dyspnea is a reflex response of the respiratory centers, de-

Chart VI.—The effect of aminophyllin on the respiratory systolic fluctuation with variable relief.

Chart VII.—The effects of epinephrine and of aminophyllin on the respiratory systolic fluctuation in an asthmatic patient refractory to treatment.

pendent primarily upon the CO_2 content of the blood and secondarily on O_2 saturation. If oxygen is administered, the patient will experience some subjective relief even though the bronchial obstruction is not released. The asthmatic type of breathing persists, because if it did not, CO_2 would accumulate and soon stimulate the respiratory centers to further activity. Aminophyllin is said to produce vasodilatation of the coronary arteries (F. M. Smith, et al.,[6] Essex, et al.[7]) and of the renal arteries (Deschamps[8]) and to increase the total blood flow through the pulmonary circulation (Mitchell[9]). The last action would expose more blood per minute to the alveolar membrane and so promote more rapid elimination of CO_2 and greater oxygenation of the blood. The respiratory centers would be subjected to the lesser stimulus of a lower blood CO_2 content; the anoxia would be reduced. The patient would then experience an appreciable diminution of his dyspnea, even though the bronchial obstruction was only partially released.

An alternative supposition, from a purely speculative point of view, is that part of the physiologic response in human beings which eventuates in bronchial asthma may be a pulmonary arteriolar spasm, similar except for reversibility to that occurring in rabbit anaphylaxis. The obliteration of this arteriolar spasm by the vasodilating action of aminophyllin would then explain in part the relief of asthma by this drug. This hypothesis invites further study.

In either case, an improved blood flow throughout the pulmonary circuit appears to us to be an important, if not the principal, factor in the relief of asthma by aminophyllin.

Our interpretation of the data presented here is highly theoretical and open to discussion. It raises a number of questions. Does the venous pressure fluctuate with respiration in asthma, and if so, what time relation does its fluctuation bear to the arterial fluctuation, and how is it affected by epinephrine and aminophyllin? What are the variations of the intrathoracic pressures in asthmatic patients as related to the respiratory systolic fluctuation, and to relief? What changes occur in the blood CO_2 and the arterial O_2 saturation, and in the pulmonary circulation time before and after these drugs? These are answerable only by investigation. It is hoped that others will make additional observations along these lines, testing our findings and carrying them further.

CONCLUSIONS

Following the subcutaneous injection of epinephrine, if relief from asthma is obtained, the respiratory systolic fluctuation decreases to or near the normal.

Following the intravenous injection of aminophyllin, if relief is obtained, the respiratory systolic fluctuation may decrease, but not to an extent comparable to that with epinephrine.

Following either drug, if relief is not secured, the respiratory systolic fluctuation remains wide.

It is suggested that the principal action of aminophyllin in relieving asthma is by increasing the blood flow through the pulmonary circulation by vasodilatation and that its bronchodilating effect is of secondary importance.

A generous supply of aminophyllin was furnished us by G. D. Searle & Co. for use in our studies.

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POLLEN SURVEYS IN THE UNITED STATES: A CRITICAL REVIEW*

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CAREFUL etiologic diagnosis and specific therapy of hay fever presuppose an accurate knowledge of the pollen in the air in the patients' vicinity capable of producing pollinosis, and of the seasons during which the pollen is present. Although in a given community a relatively few plants are responsible for the vast majority of pollen sensitivities, in the remaining cases it is precisely due to the pollens less common, and hence more difficult to appraise, that such information has particular value, since it makes possible the selection of the proper pollens to be used in testing. Moreover, it is a well-known fact that only a small minority of patients are hypersensitive to but one pollen. And even those physicians who elect to use an extract of only one species of pollen for the treatment of a case with multiple hypersensitivities do not deny the necessity for thorough etiologic diagnosis. Furthermore, it is often desirable to compare the symptoms of both treated and untreated patients, particularly those who fail to react to tests despite evidence of clinical hypersensitiveness, or those who react in the absence of such evidence, with available phenologic information.

For all these reasons, the accumulation of dependable data concerning the distribution and pollination times of plants causing pollinosis is a fundamental step in the clinical management of hay fever. Since the pioneer studies of Scheppegrell and Durham, a considerable store of information has been amassed. Yet, for a territory as extensive as continental United States, there are still many gaps in our knowledge. We are aware, of course, that many unpublished ob-

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Received for publication, December 29, 1942.

servations have been made, but these need not concern us here. It is the purpose of this paper to evaluate the work which has been reported and on this basis to point out how and where future studies may be performed so as to have the greatest value.

The authors undertook to summarize the published data on this subject. In all, the literature yielded 166 studies of varying scope and content. A complete list of the references classified by states and including those for Canada will be found in a recent article.[1] Pollen data may be gathered in two ways: botanical or field observations, and atmospheric pollen counts. Of the recorded investigations, over one-half are based on the former approach, about one-third on the latter, and only about a score on a combination of both.

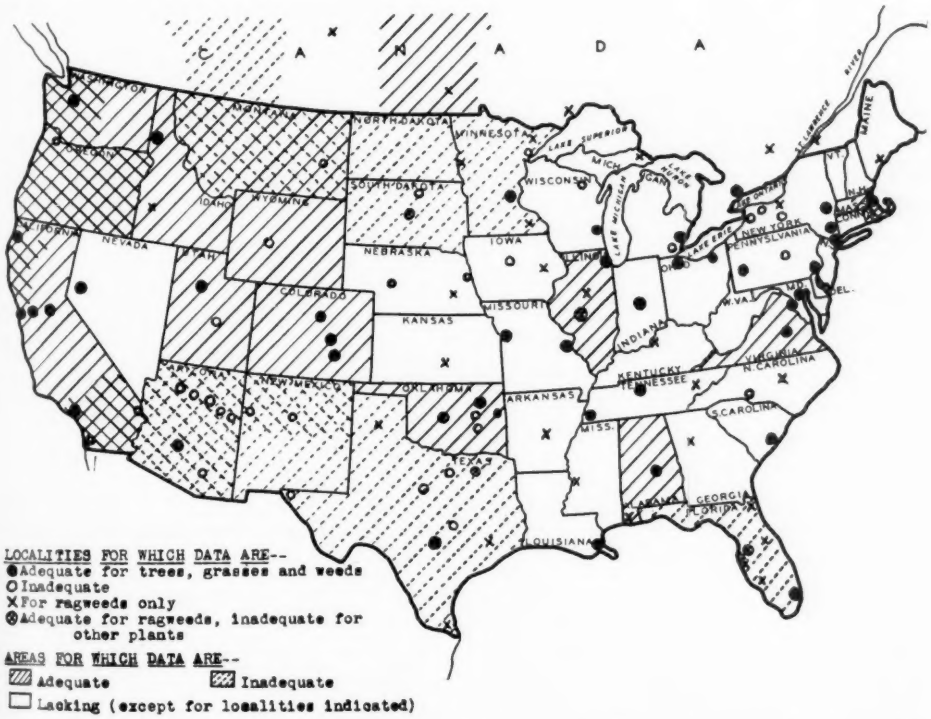

Fig. 1.—Spot map showing the localities and areas for which pollen studies have been reported. A second survey in the same region is indicated by cross-hatching.

GEOGRAPHIC CONSIDERATIONS

Pollen surveys have been reported from most sections of the United States and from some portions of Canada. Most of them concern only one city, but some include observations regarding one species made in a number of cities. Several encompass an entire state or a considerable portion of one. And a few cover several states in extent. Fig. 1 gives the exact locations and regions where such studies have been made. They are classified as inadequate if only one or a few species are considered, if over-all seasons of groups of plants are given instead of individual dates, if no dates are included, or if only a portion of the hay fever season is reported. Because so much work has been done on them

alone, the ragweed observations are indicated separately. It will be seen that, for some reason, the western portion of the country has been covered more intensively than the eastern.

The following cities, each with a population exceeding 100,000 (according to the 1940 census) have never been the scene of a pollen survey: Birmingham, Ala.; Bridgeport, Hartford, and New Haven, Conn.; Wilmington, Del.; Fort Wayne, Gary, and South Bend, Ind.; Des Moines, Iowa; Kansas City, Kan.; Fall River, Lowell, New Bedford, Somerville, and Springfield, Mass.; Flint, and Grand Rapids, Mich.; Camden, Elizabeth, Jersey City, Newark, Paterson, and Trenton, N. J.; Utica, N. Y.; Akron, Canton, Cincinnati, Columbus, Dayton, and Youngstown, Ohio; Erie, Reading, and Scranton, Pa.; Chattanooga, Tenn.; Norfolk, Va., and Tacoma, Wash. In addition, the following cities of the same size have been covered only for ragweeds: Jacksonville, Fla.; Peoria, Ill.; Wichita, Kan.; Syracuse, N. Y.; Houston, Tex.; and Spokane, Wash.

At no place in the following six states,* with a combined population of nearly nine million, have any pollination studies been carried out: Connecticut, Delaware, New Hampshire, New Jersey, Vermont, and West Virginia. If the ragweeds are disregarded, the following eight states, containing over 17½ million persons, may be added to the list: Arkansas, Georgia, Kansas, Kentucky, Maine, Mississippi, South Carolina, and Wisconsin.

Figs. 2 and 3 show directly for the ragweeds and other plants for which states information is not available or not adequate. In the evaluation here the data were considered inadequate for a state if only one city within its borders was studied, or if there was only an incomplete statewide survey. If there were studies from two or more widely separated cities within a state, or if one state wide and one city report existed, the "adequate" classification was assigned. If still more information was found, the data were listed as relatively complete.

If separate maps had been prepared for trees, grasses, and weeds other than ragweeds, the appearance of Fig. 3 would have been modified somewhat, though in the main the changes would not have been great. Table I gives the number of states falling into each subdivision for each of the major groups of plants. It will be seen that the ragweeds are by far the most widely covered, with trees the least. In addition to the states listed above, there is no information relative to the pollination dates of trees anywhere in Louisiana, Nebraska, North Dakota, Pennsylvania, and South Dakota; of grasses in Iowa, Nebraska, and North Carolina; and of weeds other than ragweeds in North Carolina.

BOTANIC CONSIDERATIONS

The reports examined varied greatly in the number of species or groups of plants included. Many involve only one, the greatest emphasis being on the ragweeds. Others consider the entire hay fever season, naming from a few to over a hundred different plants. In all, 863 species in 312 different genera are mentioned in the literature (Table II). Needless to say, not all of them bear equal weight, and in fact 392 species are referred to only once each (6 among the ragweeds, 123 among the other weeds, 169 among the grasses, and 94 among the

*We are fully aware of the fallacy of applying state borders to pollen surveys. Since a number of reports are statewide in scope, however, it forms a convenient, and indeed the only comparable, basis of comparison.

trees). These are presumably unimportant plants nationally, in relation to hay fever. As might be expected, in proportion to the number of genera or species, the ragweeds have received by far the most attention. The amount of consideration given to the other plants is surprisingly uniform. It may be noted that 5,210 pollination dates are included in 166 articles, or an average of about 31 for each. In general, one report contained the dates for a number of species in one locality, or for one species in many places. Some, of course, included many more; others only a few.

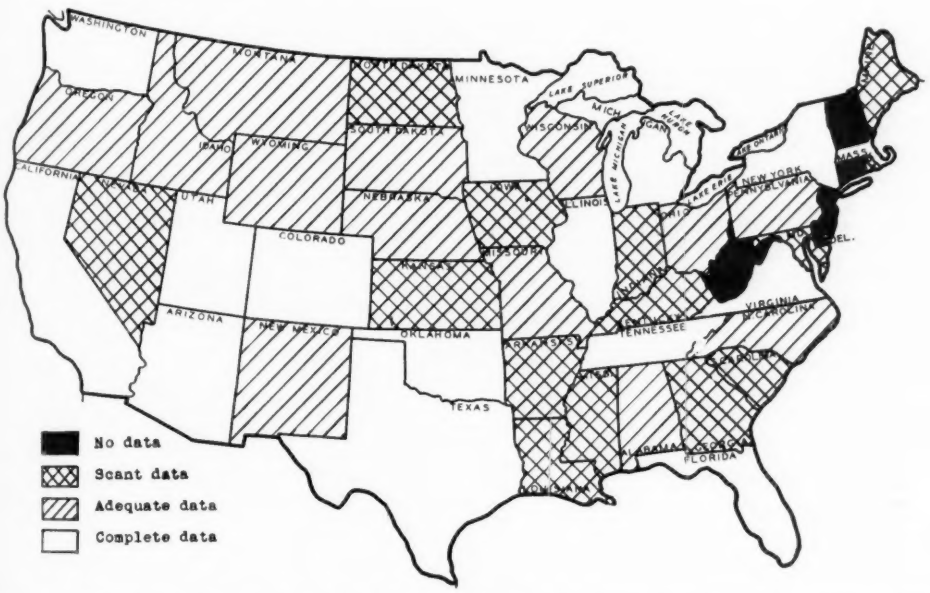

Fig. 2.—Survey of the accumulated data concerning the distribution and pollination times of the members of the ragweed family (Ambrosiaceae) by states.

TABLE I

NUMBER OF STATES WITH AND WITHOUT DATA FOR EACH GROUP OF HAY FEVER-PRODUCING PLANTS

NUMBER OF STATES	RAGWEEDS	OTHER WEEDS	GRASSES	TREES
Entirely Without Data	6	15	17	19
With Scant Data	14	14	14	9
With Adequate Data	13	9	8	10
With Fairly Complete Data	15	10	9	10

TABLE II

AVAILABLE POLLINATION DATA IN THE UNITED STATES FOR EACH GROUP OF HAY FEVER-PRODUCING PLANTS

	NUMBER OF GENERA	NUMBER OF SPECIES	NUMBER OF TIMES MENTIONED	AVERAGE NUMBER OF TIMES MENTIONED PER SPECIES
Ragweeds	4	30	748	24.9
Other Weeds	122	267	1493	5.6
Grasses	108	325	1671	5.1
Trees	78	241	1298	5.4
Total	312	863	5210	5.9

We do not for a moment maintain, either here or in the succeeding two tables, that the number of times a plant family, genus, or species is mentioned need necessarily be an index of its importance in pollinosis locally or nationally. It will be seen, however, that there is likely to be some correlation. Moreover, it is thought that these summarized statistics might be of interest in showing where the attention of the workers in this field is directed.

Table III contains a list of the most important genera, the number of species of each considered, and the total number of times each is referred to in the literature.

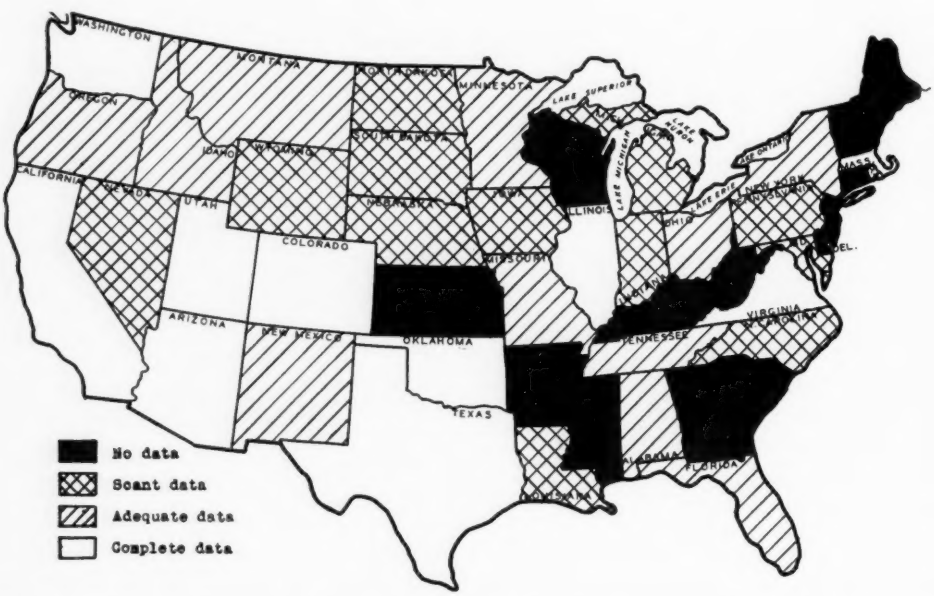

Fig. 3.—Survey of the accumulated data concerning the distribution and pollination times of trees, grasses, and weeds not members of the ragweed family, by states.

Table IV lists the individual species named most often. While notations of the "ragweeds" without differentiation of the specific representatives by far exceed all the rest, it is interesting to note that three grasses and two weeds surpass the leading ragweed. The sum of the figures for the three ragweeds included, however, reaches an impressive total. It will be seen that only four trees are to be found in the table. This is merely a reflection of the fact that many studies give dates only for the genera of trees rather than for each type, and that the statements in others are divided rather uniformly between a number of trees in each genus. Hence, few individual species were able to accumulate any considerable total.

Finally, it not infrequently occurs that a comparison of the pollination dates reported by two or more investigators for the same place reveals considerable discrepancies. For instance, the season for elm (*Ulmus*) in San Francisco is given by Rowe[2] as January and February, and by Deamer and McMinn[3] as the second week in March to the end of April; for orchard grass (*Dactylis glomerata*) in Richmond, Virginia, by Vaughan, Graham, and Crockett[4] as February 23 to June 14, by Blanton[5a] as June 6 to June 23, and by Vaughan[5b] as June

and July; and for giant ragweed (*Ambrosia trifida*) in San Antonio, Texas, by French[6] as July 29 to mid-December (first frost), and by Kahn[7] as mid-September to the end of November. Many other similar examples could be cited. These discrepancies may be explained by differences in method, by the fact that the studies were conducted in different years, or by other factors. In many instances, however, there appears no direct statement as to the techniques employed; the year in which the work was done; whether the season observed is to be taken as typical for the locality; and other pertinent information that is essential if adequate comparisons are to be made between surveys by different investigators in the same area. Variations in the stated prevalence of plants are as great or greater.

It is beyond the scope of the present paper to discuss in detail the techniques for determining pollination data. An evaluation of present knowledge, however, requires some consideration of the methods by which they are obtained. Since certain unavoidable sources of error inherent in these procedures are discussed elsewhere,[1] they need only be enumerated here. In brief, they include variations from year to year in the pollination dates of a given species, and in the relative abundance, pollinating capacity, and clinical importance of different plants, depending on meteorologic conditions; effects of the local growth of cultivated or uncultivated species over a small area; unusual conditions of exposure; and the influence of altitude on the pollination dates and nature of the flora even within a comparatively limited region. In addition to these factors, however, there are in many studies certain deficiencies which could be rather easily controlled by a careful application of, or slight modification of, the method employed. The nature of these shortcomings and the means by which they might be overcome may best be discussed separately for each type of investigation.

BOTANICAL SURVEYS

In the first place, casual botanical surveys are of little value, since the errors of random sampling may easily affect the findings to a great degree. Only when the region under consideration is painstakingly analyzed from the botanical standpoint and an adequate number of "stations" designated, will the results be significant. Then, each station must be frequently and regularly visited, and detailed notations made of the relative frequency of each anemophilous species, the date of onset and termination of pollination, and the abundance of pollen produced. To insure that the precise dates of each important species can be recorded, the observations must be made at sufficiently short intervals. The average figures for all the stations may be considered characteristic for the area studied. Examples of investigations so performed include, among others, the surveys of Chicago by Koessler and Durham,[8] of the eastern shore of San Francisco by Rowe,[9] and of Winslow and Holbrook, Arizona, by Lamson and Watry.[10]

In this connection, it may be noted that there is no scientific justification for assuming, as many authors do, that the findings in one community are necessarily representative for the surrounding region, the state, or a considerable section of the country, unless such a conclusion is supported by observations throughout the designated area. Thus, Lamson and Watry[11] showed that four

towns in northern Arizona, although only from 42 to 66 miles apart, had very few species of plants in common. Comparable observations have been made by Sellers[12] in Texas, Mullin[13] in Colorado, and by others elsewhere.

TABLE III

GENERA MOST FREQUENTLY MENTIONED IN THE LITERATURE

GENUS	NUMBER OF SPECIES	NUMBER OF TIMES MENTIONED
"Ragweeds":		
Ragweed (Ambrosia)	7	479
Marsh Elder, Poverty Weed (Iva)	8	114
Cocklebur (Xanthium)	7	83
False Ragweed (Franseria)	8	71
Other Weeds:		
Sagebrush, Wormwood (Artemisia)	27	244
Goosefoot (Chenopodium)	16	160
Pigweed (Amaranthus)	9	149
Saltbush, Orache (Atriplex)	27	140
Dock, Sorrel (Rumex)	15	137
Plantain (Plantago)	9	122
Russian Thistle (Salsola)	1	84
Burning Bush (Kochia)	4	48
Water Hemp (Acnida)	2	25
Nettle (Urtica)	6	22
Sunflower (Helianthus)	1	21
Dandelion (Taraxacum)	2	20
Goldenrod (Solidago)	4	19
Hemp (Cannabis)	1	19
Greasewood (Sarcobatus)	1	18
Winter Fat (Eurotia)	1	17
Grasses:		
June, Blue (Poa)	10	185
Brome, Chess (Bromus)	20	105
Wheat Grass, Quack (Agropyron)	12	89
Timothy (Phleum)	1	84
Red Top, Bent (Agrostis)	9	79
Orchard (Dactylis)	2	72
Fescue (Festuca)	13	60
Wild Rye (Elymus)	8	55
Wild Barley, Foxtail (Hordeum)	8	53
Ray Grass (Lolium)	4	53
Bermuda (Cynodon)	1	52
Johnson (Holcus)	4	43
Barnyard (Echinochloa)	4	33
Oats (Avena)	3	29
Salt Grass, Spite (Distichlis)	2	29
Panic Grass (Panicum)	17	27
Sweet Vernal (Anthoxanthum)	1	26
Smut Grass, Rush Grass (Sporobolus)	6	24
Foxtail (Chaetochloa)	5	24
Corn (Zea)	1	24
Koeler's (Koeleria)	1	23
Trees:		
Oak (Quercus)	31	163
Poplar, Cottonwood (Populus)	20	140
Maple, Box Elder (Acer)	10	109
Elm (Ulmus)	8	86
Willow (Salix)	20	68
Birch (Betula)	10	64
Juniper, Cedar (Juniperus)	8	62
Ash (Fraxinus)	11	59
Pine (Pinus)	11	59
Hickory, Pecan (Carya)	8	54
Walnut (Juglans)	5	54
Alder (Alnus)	6	42
Sycamore (Platanus)	4	36
Hazel (Corylus)	3	27

Certain deficiencies encountered in field surveys could be readily avoided. Many reports identify plants only by common names, which are often ambiguous and overlapping. Use of the technical nomenclature is far more precise and has the additional advantage of indicating botanical relationships. Some studies group considerable numbers of plants into rough seasons, such as "spring" or "fall," rather than giving the separate dates for each; others specify pollination times only by months, although greater accuracy would be desirable. The amount of consideration, if any, given to the prevalence of different plants varies tremendously, as does the explanation of the procedures by which the data are gathered.

Reference to herbarium specimens to determine the distribution and pollination times of plants, while it was of use in the early period of pollen studies, is obviously inadequate and requires no further discussion.

But even when most carefully performed and recorded, the botanical survey by its very nature must still be a comparatively crude method, particularly in estimates of the prevalence of the plants and the abundance of pollination. And even when only anemophilous species are considered, it gives no information as to how far the pollens are carried by the wind and whether they ever reach the vicinity of any considerable number of people.

TABLE IV

SPECIES MOST FREQUENTLY MENTIONED IN THE LITERATURE

SPECIES	NUMBER OF TIMES MENTIONED
"Ragweeds" (species not identified)	288
Russian Thistle (*Salsola pestifer*)	84
June Grass (*Poa pratensis*)	84
Timothy (*Phleum pratense*)	84
Lamb's Quarters (*Chenopodium album*)	70
Orchard Grass (*Dactylis glomerata*)	68
Dwarf or Short Ragweed (*Ambrosia elatior*)	66
English Plantain (*Plantago lanceolata*)	61
Red Top (*Agrostis alba*)	59
Red Root Pigweed (*Amaranthus retroflexus*)	59
Giant or Tall Ragweed (*Ambrosia trifida*)	57
Bermuda Grass (*Cynodon dactylon*)	52
Burweed Marsh Elder (*Iva xanthifolia*)	49
Western Ragweed (*Ambrosia psilostachya*)	48
Annual Blue or Low Spear Grass (*Poa annua*)	40
Sheep Sorrel (*Rumex acetosella*)	39
Box Elder (*Acer negundo*)	38
English or Perennial Ray (Rye) Grass (*Lolium perenne*)	38
Curly or Sour Dock (*Rumex crispus*)	38
Johnson Grass (*Holcus halepensis*)	34
Canada Blue Grass (*Poa compressa*)	32
Quack or Couch Grass (*Agropyron repens*)	32
Burning Bush, Firebush (*Kochia scoparia*)	30
Small Poverty Weed (*Iva axillaris*)	29
Sagebrush (*Artemisia tridentata*)	28
False Western Ragweed (*Franseria acanthicarpa*)	28
Meadow Fescue (*Festuca elatior*)	28
Mugwort, Wormwood (*Artemisia vulgaris*)	27
Common Plantain (*Plantago major*)	27
Salt Grass (*Distichlis spicata*)	26
Sweet Vernal Grass (*Anthoxanthum odoratum*)	26
Barnyard Grass (*Echinochloa Crus-galli*)	25
Spearscale, Hastate Atriplex (*Atriplex hastata*)	24
Corn (*Zea mays*)	24
Cocklebur (*Xanthium canadense*)	22
Foxtail Grass, Wild Barley (*Hordeum jubatum*)	21
Giant Wild Rye Grass (*Elymus condensatus*)	21
White Elm (*Ulmus americana*)	20
Giant Poverty Weed, Rough Marsh Elder (*Iva ciliata*)	19
Hemp (*Cannabis sativa*)	19
Pasture Sagebrush, Mountain Sage (*Artemisia frigida*)	18
Black Walnut (*Juglans nigra*)	18
Cottonwood, Carolina Poplar (*Populus deltoides*)	17

POLLEN COUNTS

The pollen count or the direct determination and identification of the pollens in the air, then, is free from these two objections, since it is a quasi-quantitative measure of the actual quantity of the allergen (assuming each pollen grain of the same species to contain an equal amount) in the immediate proximity of one patient or a given population group, depending on the place of exposure. The

simplest technique merely allows the air-borne pollens to fall by gravity to the surface of a microscopic slide coated with a viscous substance, and the number caught on a fixed area (e.g., one square centimeter, one square inch, one square foot, or 1.8 square centimeters) during a unit time, usually one day, is counted and calculated. At the same time, they may be identified within the limits of the botanical differentiation of pollens by comparing them with their known characteristics[14-19] or with a reference collection of the different kinds. Needless to say, such "differential counting" of the pollens yields far more useful data than are obtained from total counts.

The morphology of the grains will vary depending on the adhesive (petrolatum, glycerin, glycerin jelly, corn, linseed, or cedar oil[20]) selected to apply to the slides. Likewise, stains (Lugol's solution, methyl green, Caberla's solution, aqueous or basic fuchsin, aqueous eosin, or nigrosin) or a contrast color method[21] may be used, if desired, to modify the microscopic appearance. Such variations in technique are at the choice of the investigator, who should, of course, be familiar with their effects. They may, in some instances, facilitate the identification, but it should be recognized that they do not in any way affect the actual findings nor lead to more minute differentiation. Recently, specially prepared slides have been suggested[22, 23] to speed the counting and to ensure accurate measurement of the surface area covered.

Various types of apparatus[24-28] have been advocated to obviate the effects of gravity. It is of interest to note that Blackley himself over sixty years ago devised an impingement method. While they have the advantage of permitting the collection of the pollen to be completed in a short period of time and thereby of detecting rapid fluctuations in the degree of atmospheric pollen pollution, they are too cumbersome for widespread practical application and provide no more information utilizable clinically than do the usual techniques. Studies based on slides exposed during airplane flights[29-32, 26] have increased our knowledge of the behavior of pollens in the upper air, but are likewise not clinically applicable.

Probably the greatest single disadvantage of the pollen-counting method is the inability to differentiate between related types of pollen grains. For example, the pollens of all the grasses are identical or nearly so. Likewise, all the members of the family Ambrosiaceae, including the various ragweeds, false ragweeds, marsh elders, and cockleburs must perforce be grouped together, as do those of the order Chenopodiales, containing the pigweed (Amaranthaceae) and goosefoot (Chenopodiaceae) families, since they are microscopically indistinguishable. This constitutes, of course, a serious obstacle to precise work in this field. It may be hoped that by means of differential stains or other techniques, a method will be developed in the future to obviate this difficulty. The pollens of the various genera of trees, on the other hand, are morphologically distinctive.

Most pollen counts have been performed in cities. Just as with field surveys, it is necessary for best results to utilize a number of carefully chosen stations, since differences of terrain, elevation, the presence of tall structures, variations in the flora of the outskirts and in prevailing winds will greatly affect the findings. Such multiple station pollen counts may be exemplified (the list being

by no means complete) by the studies of Koessler and Durham[8] in Chicago, Rowe[9] in the San Francisco Bay area, Acquarone and Gay[33] and Patterson and Gay[34] in Baltimore, and Deamer and McMinn[3] in San Francisco. There is little to be gained by emphasizing the differences between closely situated stations, but the figures for all of them may well be averaged for the community.

The intensive pollen studies by O. C. Durham in conjunction with the Weather Bureau over a number of years in many of our large cities have resulted in the accumulation of a considerable fund of information. Unfortunately, they have practically all been confined to the "ragweeds," including all members of the family, without consideration of other plants. While ragweed unquestionably is our most important plant causing pollinosis, only a small minority of fall hay fever victims are hypersensitive to its pollen alone; most of them are also allergic to other species. Moreover, in the interests of scientific accuracy, it is highly desirable to break down the family into its individual representatives in order to correlate the patients' symptoms with the pollen content of the air, to test adequately, and to treat specifically.

The authors have observed that the figures for ragweed as issued by the Weather Bureau often appear lower than the symptoms of the sufferers would seem to warrant. This is presumably due to the fact that the slides are exposed at the top of a tall building in the center of a built-up metropolitan area, an ideal place for meteorologic observations, rather than at the ground level (or more precisely, the "breathing level") in the residential sections, where the counts may be assumed to be considerably higher because of the effects of gravity and the proximity to fields and vacant lots. This illustrates the importance of choosing the sites of exposure with care, and it may be advisable, as well, to include neighboring suburban areas for similar reasons. In short, it would be advantageous to place the slides where the majority of the population spend the bulk of their day.

It has been customary in most of the reports to give graphs or tables of the daily fluctuations of the pollen counts. While this is informative, clinically it is only of minor interest in the individual case, since every sufferer and every physician is already well aware that there will be variations in the severity of the symptoms from day to day, and indeed, from hour to hour. Moreover, because the curves vary so greatly from year to year (for example, see Durham[35]), the experience of previous seasons cannot be expected to hold in the future. Hence, these quantitative considerations are of little value as compared with the simple knowledge of the species of pollen present and the exact beginning and end of the pollination of each.

The gravity method has been criticized as giving no real measure of the number, or weight, of pollen grains per unit volume of air, since each species of pollen falls at a different rate. Various formulas have been devised to obtain a volumetric value by applying to the pollen counts correction factors based on the size and shape of the grains, as well as on the surface irregularities of those which are echinated, winged, or sculptured. Scheppegrell[36] first made such an attempt based on Stokes' formula. Errors in his application of the physical principles were discovered by Cocke,[37] and mistakes in the calculations of the latter were pointed out by Dahl and Ellis.[38] The last-mentioned and

Durham[39] have also emphasized the equal significance of variations in the weight and specific gravity. There have been some differences of opinion (Cocke,[27, 37] Durham,[39] Hawes, Small, and Miller,[28] and Dahl and Ellis[38]) as to the advantage or disadvantage of employing mathematical correction factors so as to obtain a more accurate index of the number of pollen grains contained in a unit volume of air. Without entering into the merits of the various claims, one may point out that the entire question is more of academic than of practical import. Sufficient difficulties are encountered in the formulas involving size alone, disregarding the fact that pollen grains of the same species vary somewhat in diameter. But in regard to the effect of spiculation (as with ragweed pollen) or other irregularities of contour on the rate of fall, and hence on the volumetric computations, only rough estimates rather than reliable calculations are available. The same statement holds for differences in specific gravity. With any one species, the twenty-four-hour "gravity count" is an adequate guide to daily fluctuations and to comparisons of the pollen density at one locality with that at another. Furthermore, there is so much variation in the antigenicity of different pollens and in the respective tolerance of individual patients that a comparison of the relative numbers of the various species, even if perfectly accurate, would have no clinical significance. Moreover, it has been amply demonstrated that the inequalities in the counts at two or more locations within the same city, indeed, on different floors of the same building (Wilmer and Cobe[40]), may be far greater than the effect of such corrections. Vaughan,[41] in discussing the attempts at the standardization of pollen counting, concluded that for practical purposes, including comparison of the concentrations in different years, it is sufficient to record the number of grains per unit area, rather than to estimate mathematically the number per cubic yard per twenty-four hours. Certainly, none of the more recent studies gives any reason to abandon this viewpoint. Finally, while the greatest possible exactness in all aspects of this subject is desirable, simple but reliable data on the duration of pollination would far outweigh all quantitative statistics, whether on a volumetric or a unit area basis.

COMMENT

On the basis of the facts given above, what recommendations can be made concerning the most effective means of increasing our knowledge of phenologic phenomena as they relate to hay fever? It must be apparent that neither field studies nor pollen counts alone are adequate. Both must be simultaneously performed at numerous, carefully chosen stations in the given city or region and the findings correlated. In this way, they supplement each other, so that a rise in the total grass pollen count, for example, can be properly attributed to the particular species observed to be pollinating at that time. Contrariwise, the counts act as a check on mistaken conclusions as to the abundance, profuseness of pollination, or buoyancy of the pollen of various plants, as determined by botanic observations. The identification of both plants and pollens must be accurate, and scientific names employed. The exact technique and the unit of recording of the counts are in themselves of relatively little importance, but adherence to a single unit would obviously be highly advantageous. If correction factors are used, they should be clearly stated, and the basis of the computations

given. There is little to be gained by listing every anemophilous species in a locality, since in the present state of clinical knowledge many of them are of no significance. Too much emphasis should not be placed on daily variations in the count, for, as outlined above, this information is of little practical value. The stress should rather be on the determination of the type of pollen in the vicinity of potential or actual hay fever sufferers, just when each first appears and when each can no longer be found in the air. Since the pollination season varies from year to year,[1] such studies should be continued for a number of successive years so as to accumulate information, first, as to the usual or average dates, and, second, as to the amount of variation which occurs or is likely to occur. Data of this nature are immediately useful in selecting pollens for testing and treatment, in evaluating patients' symptoms, and in planning preseasonal, coseasonal, and perennial therapy.

Rosendahl, Ellis, and Dahl[42] summarize the requisites as follows: "From a clinical standpoint, it is of importance that pollen data demonstrate the following facts: 1. The approximate time (variability is not great) at which a specific kind of pollen annually makes its appearance in the air and when it reaches clinically significant concentration. 2. The duration of the period for which clinically significant amounts are present. 3. The dates (approximate) at which the concentration drops below the level of clinical significance."

While agreeing in principle, we must point out that there is no present method of determining the clinically significant concentration of pollen. While, on a rough statistical basis, the symptoms of a large group of sufferers parallel the count for their specific pollen, the correlation is a crude one, and a considerable percentage of patients seem to run contrary, or at least independently during the season, particularly in its latter portion. There is every reason to believe that the pollen incidence capable of eliciting symptoms will vary greatly for different patients; for the same patient from time to time; for different species; and will change according to the state of the offending pollen grains (dry or moist, fresh or old).

But even the most accurate, most reliable pollen data would still leave much to be desired in our evaluation of the importance of each species in pollinosis. It is a well-known fact that a pollen may be abundant and buoyant, as for example that of pine, and still give rise to little or no hay fever. This, of course, corresponds to Thommen's[43] first postulate. Only by the simultaneous collection of pollination data and the clinical investigation of large groups of patients in the same area will any basis be reached for judgment. For this purpose, statistical studies of skin reactions suggest themselves as the most readily applicable and simplest method. But skin tests alone are inadequate, since cutaneous and clinical hypersensitiveness need not always coincide. It is now recognized that a certain proportion of pollinosis cases give positive cutaneous responses to species which elicit no other allergic manifestations in them, and even to those with which, for geographic reasons, they could never have come into contact. Skin reactions should therefore be compared with the presence or absence of symptoms during the pollinating period of the given plant. In addition, nasal and conjunctival tests may often be profitably resorted so as to confirm the reaction with that of other tissues. It is likewise well-known that

some cases will fail to react to the pollens to which they are hypersensitive. Once the diagnosis of hay fever is established in such cases, the determination of the etiologic agent will depend on careful evaluation of the entire clinical picture, with special emphasis on the patients' "seasons," fluctuations in symptomatology as related to variations in pollen pollution, the response to specific therapy, and the inadvertent elicitation of constitutional reactions with specific extract. The possible difficulties encountered in evaluating the results of tests and in establishing the etiologic diagnosis are discussed by the authors[44] elsewhere. Despite these problems, it is only by a correlation of concurrent phenologic and clinical data that any safe conclusions can be reached as to the relative importance in pollinosis of each species of tree, grass, or weed.

Where may such pollen studies most profitably be performed? If the intention is merely to fill in the existing gaps, a glance at the maps and lists included will provide the answer; however, a coordinated nationwide survey combining botanic observations, pollen counts, and clinical investigations, according to a uniform technique, would yield a mass of valuable information and go far to resolve the present confusions and contradictions in this subject. The equipment required and the cost of such a study would be negligible in comparison to the utilizable knowledge it would provide. It could most advantageously be carried out in the larger centers of population, since there the great majority of hay fever victims reside, and since only there would the necessary facilities be found, along with a sufficient number of patients, to make the clinical data significant. As mentioned, the investigation would have to be continued for a number of successive years.

Very recently the Society for the Study of Asthma and Allied Conditions[45] published a preliminary record of the pollen surveys by their members. This is, indeed, the first collective effort to solve the problem under consideration, and will, when all data are published, undoubtedly prove a major contribution to the aim outlined above.

While the actual work in a study such as the one suggested could be done by allergists and botanists in the various communities, obviously a nationwide organization would be necessary to lay down standards, to decide on a uniform technique, and above all, to analyze and correlate the accumulated data so as to reach clinically useful conclusions. The logical choice for this purpose would be one of the national allergy societies or the Public Health Service.

SUMMARY

A review of the existing fund of information regarding the distribution and pollination times of the pollinosis-producing plants in the United States reveals: (1) many states and large cities where no data or only inadequate data are available; (2) certain unavoidable sources of error in the methods of investigation; (3) common correctable deficiencies in the published reports which greatly diminish their value; (4) an unwarranted emphasis, in some instances, on large numbers of species, rather than on those of clinical importance; and (5) an emphasis on quantitative considerations unjustified by present clinical knowledge or applicability.

A correlated pollen survey based on simultaneous botanical observations, pollen counts, and clinical investigations is suggested. This should be done on a nationwide scale and according to a uniform technique.

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RELATION OF LENGTH OF CARBON CHAIN TO THE PRIMARY AND FUNCTIONAL TOXICITIES OF ALCOHOLS*

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IN A previous publication[1] a method was described for determining the toxicity of antiseptics on human or guinea pig blood as a tissue by measuring inhibition of phagocytic activity. Later work[2] showed that in many instances the phagocytic inhibition occurred through destruction of some component or components of the humoral system, while in others the leucocyte itself was destroyed. Following a study[3] of the use of other components of the blood as a measure of toxicity, a new test was described[4] in which toxicity was measured by actual death of the cell (leucocyte). Both of these tests were developed primarily to determine the direct toxic effect, mainly of antiseptics, on whole blood as a tissue. Such tests may be used to determine the "primary toxicity" of a single compound or the "relative primary toxicities" of a group of compounds.

The term toxicity has come to have a broad meaning. It is used to describe not only primary "irritating," "damaging," or "destructive" action on an isolated tissue, as used for instance by Salle et al.[5] in his toxicity test on chick embryo tissue or by Bronfenbrenner et al.[6] with his toxicity method using mouse liver cells, but also is used to designate what might be called the "functional" toxicity of a chemical injected into or fed animals in the assay of such compounds. Hence Macht[7] describes the "toxicity" of the primary alcohols for cats when injected intravenously, while Woodard and his collaborators[8] report on the "acute oral toxicity" of acetic and the chloracetic acids. *In contrast to the "primary toxicity" of a compound on tissue cells in vitro which measures*

*From the U. S. Food and Drug Administration, Washington, D. C.
Received for publication, January 7, 1943.

a single isolated toxic action, measurement of "functional" toxicity not only encompasses the sum of the primary toxic effects, but also includes other factors such as the selective affinity of some chemicals for certain organs, cells, or cellular mechanisms of the animal body and the physiologic effects of end products of metabolism of the chemical. Thus where "primary" toxicity is tested on human blood in vitro, using inhibition of phagocytic activity as an index of toxic effect, phagocytosis ceases in the presence of 5 per cent of methyl alcohol. Yet when methyl alcohol is taken into the digestive tract, products probably produced by intermediate oxidation bring about a deleterious effect in the animal which bear no relation to the "primary" toxic effect measured on individual cells. Similarly potassium cyanide which produces death when injected or fed in small amounts has very little effect on the isolated white cell even in high concentrations.

In the present study the primary toxicities of the monohydroxy aliphatic alcohols and the toxicities of acetic, mono-, di-, and trichloracetic acids have been determined by the method previously described,[1] using inhibition of phagocytosis in whole human and rat blood in vitro as an index of toxic effect. These toxicities have been compared with the acute oral toxicities obtained on the acids by Woodard, Lange, Nelson, and Calvery[8] and on the alcohols by Woodard.* The rats used were predominantly males and were raised by the Division of Pharmacology of the Food and Drug Administration. They ranged in weight from 150 to 250 Gm. and in age from 70 to 100 days. The alcohols were administered to the rats orally after an eighteen-hour fasting period by means of a soft rubber catheter attached to a syringe. Following treatment the rats were observed for six days, and those surviving were not used in subsequent experiments.

The results obtained in testing the resistance of both human and rat leucocytes to the toxic action of seven normal alcohols and the acute oral toxicity for rats of these same alcohols are given in Table I. It will be noted that as the molecular weight of the alcohols increases, the concentration of the alcohol necessary to bring about complete inhibition of phagocytosis decreases. Whereas methyl alcohol inhibited phagocytosis in a dilution of 1:20, ethyl alcohol prevented phagocytosis in a dilution of 1:30, propyl at 1:70, butyl at 1:175, amyl at 1:450, hexyl at 1:1000, and heptyl at 1:2500. The human and rat leucocyte both showed approximately the same resistance to the toxic action of the alcohols throughout the series. It will also be noted (Table I) that the molarity of the toxic dilution decreases from M/.64 for methyl alcohol to M/290.7 for heptyl alcohol in a fairly regular sequence. When the toxic molar concentration of ethyl alcohol is taken as unity and the relationship of the other six alcohols computed, it will be noted that the toxicity in this homologous series of alcohols for the leucocytes of humans or rats increases in the order of molecular weights following semiquantitatively the geometric progression described by Kamm[9] for these alcohols, $1 : 3^1 : 3^2 : 3^3 : 3^4 : 3^5$.

This generalization, i.e., that toxicity in a homologous series increases with the molecular weight, known as Richardson's Rule, has been verified by Morgan and Cooper[10] and by Tilley and Schaffer[11] in testing the germicidal action of

*The authors are indebted to Geoffrey Woodard for the unpublished data on the acute oral toxicities of the primary alcohols in rats.

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the aliphatic alcohols, by Macht (loc. cit.) in a study of the acute toxicity of these alcohols when injected intravenously in cats and by Kamm (loc. cit.) who tested their toxicity for paramecia as well as their albumen-coagulating power. In contrast to these results when acute oral toxicity is determined in rats (Table I), there is no regular relationship between toxicity and molecular weight. The alcohols increase in functional toxicity irregularly from methyl through butyl alcohol and then decrease in toxicity from amyl to heptyl alcohol. These results emphasize the essential difference between "primary" and "functional" toxicity. In the case of the latter, the fate of the animal is determined by a variety of factors including the solubility, rate of absorption and excretion, and the effect of body fluids on the alcohol.

TABLE I

TOXICITY OF PRIMARY ALCOHOLS

ALCOHOL	TOXICITY AS MEASURED BY INHIBITION OF PHAGOCYTOSIS IN WHOLE BLOOD*			ACUTE ORAL TOXICITY IN RATS†
	TOXIC DILUTION	MOLARITY OF TOXIC DILUTION	RATIO MOLARITY TOX. DIL. TO MOLARITY TOX. DIL. OF ETHYL ALCOHOL	GM. PER KG. OF BODY WEIGHT
Methyl	1:20	M/.64	.46	9.1
Ethyl	1:30	M/1.38	1.00	7.4
Propyl	1:70	M/4.21	3.05	3.3
Butyl	1:175	M/12.96	9.40	2.75
Amyl	1:450	M/39.82	28.85	3.3
Hexyl	1:1000	M/102.04	73.98	4.1
Heptyl	1:2500	M/290.7	210.76	Approx. 6.6

*Both human and rat blood were used in these tests. Equal resistance of leucocytes to the toxic effect of these alcohols was demonstrated.

†Acute oral toxicity was determined by Geoffrey Woodard, Division of Pharmacology, U. S. Food and Drug Administration.

In order to demonstrate further the difference between what has been designated in this report as "primary" toxicity for an isolated tissue or cell and "functional" toxicity, a study has been made of acetic acid and the chloracetic acids in the same manner as the alcohols were investigated. The ability of acetic acid and the chloracetic acids to inhibit phagocytosis completely in whole human and rat blood has been determined in both acid and neutral solutions, and these results compared with the acute oral toxicity in rats as determined by Woodard et al. (loc. cit.). The results are shown in Table II, in which it will be noted that acetic acid is the most toxic and monochloracetic, dichloracetic and trichloracetic acids decrease in toxicity in that order for both human and rat leucocytes. The rat leucocyte is more susceptible to the action of these chemicals than the human. The pH of the toxic dilution decreases from 3.18 for acetic acid to 1.73 for trichloracetic acid with human cells and from 3.23 to 1.85 with rat cells indicating that the toxicity is probably related to the undissociated molecule rather than to the acidity. Computation of the molarity of the toxic dilution of all four acids, i.e., that dilution causing complete inhibition of phagocytosis, indicates that within experimental error all of these acids are toxic in equimolecular concentrations.

Since it was advisable to determine the acute oral toxicities of the acid radicals and not their toxicities as acids, the acids were neutralized with sodium

hydroxide to a pH range between 6 and 7. Neutral solutions of this type were also tested for their primary toxicity for both human and rat leucocytes. These results are also given in Table II where it will be noted that the same dilution (1:70) of all four neutralized acids inhibited phagocytosis of the human leucocyte, while a 1:100 dilution of each acid prevented phagocytosis by the rat leucocyte. In direct contrast to the equivalent primary toxicities of these four neutral acids for the rat white cell, are the results obtained on feeding these acids to rats, since it will be noted that monochloracetic acid is by far the most toxic, dichloracetic the least toxic, while acetic and trichloracetic have approximately equal "functional" toxicities.

TABLE II

TOXICITY OF ACETIC AND CHLORACETIC ACIDS

ACID	HUMAN BLOOD				RAT BLOOD				RATS
	ACID SOLUTIONS			NEUTRAL SOLUTIONS	ACID SOLUTIONS			NEUTRAL SOLUTIONS	NEUTRAL SOLUTIONS
	TOXIC DILUTION	PH TOXIC DILUTION	MOLARITY TOXIC DILUTION	TOXIC DILUTION	TOXIC DILUTION	PH TOXIC DILUTION	MOLARITY TOXIC DILUTION	TOXIC DILUTION	*ACUTE ORAL TOXICITY IN GM./KG. OF BODY WEIGHT
Acetic	1:550	3.18	.03	1:70	1:700	3.23	.024	1:100	3.31
Monochloracetic	1:400	2.27	.026	1:70	1:650	2.46	.016	1:100	.0762
Dichloracetic	1:300	1.77	.026	1:70	1:400	2.00	.019	1:100	4.48
Trichloracetic	1:250	1.73	.025	1:70	1:300	1.85	.020	1:100	3.32

*Values obtained by the Division of Pharmacology, U. S. Food and Drug Administration, J. Indust. Hyg. & Toxicol. 23: No. 2, 1941.

COMMENT

The method described previously[1] for measuring the toxicity of antiseptics or other chemicals brought into direct contact with tissue, i.e., by determining that concentration of the compound under test which completely inhibits phagocytosis, may be considered to be an estimation of "primary" toxicity on an isolated cell. This toxic action may manifest itself by a destructive effect on some component or components of the humoral system or by actual destruction of the cell itself.[4] In either case the toxic action measured is on an isolated individual tissue cell. When the primary aliphatic alcohols are tested by this technique, a definite and uniform increase in toxicity is observed with increase in molecular weight. These results are in accord with those of Kamm (loc. cit.), who found the toxicity of the alcohols for paramecia to be in proportion to their molecular weight, while similar results were obtained by Tilley and Schaffer (loc. cit.) in determining the destructive action of these alcohols for *Eberthella typhosa* and *Staphylococcus aureus*. From these studies with leucocytes, protozoa, and bacteria, it is evident that when the normal alcohols are tested for their primary toxicity for individual cells, a definite relationship between molecular weight and toxic activity can be readily demonstrated.

Similarly Macht (loc. cit.) demonstrated an increase in acute toxicity with increase in molecular weight when these alcohols were injected intravenously into cats, although the increase in toxicity with increased molecular weight does

not appear to follow a definite mathematical pattern. Thus Macht reports n-propyl alcohol to have a toxicity of 2.5 times and n-butyl 16.6 times the toxicity of ethyl alcohol. Kamm (loc. cit.) points out that the abnormalities in Macht's results for n-butyl alcohol, i.e., the fact it does not follow the geometric progression $1 : 3^1 : 3^2 : 3^3$, may be ascribed to a too concentrated solution of this material when injected intravenously. It may be, however, that the abnormalities noted with butyl alcohol injected intravenously into cats are associated with the method of test, and the discrepancies, an indication of the selective action of this alcohol for some vital organ. When acute oral toxicity is measured in rats, an entirely different result is obtained. The toxicities of the first four normal alcohols follow Richardson's rule, while the last three tested failed to do so. In fact, amyl, hexyl, and heptyl alcohols decreased in toxicity in that order. Undoubtedly the lower solubilities of the higher alcohols have a marked effect on oral toxicity.

When a comparison is made of the primary toxicity, i.e., toxicity for rat tissue and the acute oral toxicity of the neutralized acetic acid and chloracetic acids for rats, we again note a marked difference in the results obtained. While monochloracetic acid has an acute oral toxicity approximately 40 times that of the other three acids, the primary toxicity for rat leucocytes was found to be the same for all four acids. These results indicate that there is a marked difference between "primary" toxicity as measured by the action of a compound on an isolated tissue cell and "functional" toxicity in which the compound is fed or injected into an animal and hence influenced either by a selective affinity of the compound for some vital organ of the body or by the physiologic action of body fluids.

SUMMARY AND CONCLUSIONS

1. The primary toxicities of the monohydroxy aliphatic alcohols, acetic acid and chloracetic acids against human and rat blood as tissues have been contrasted with the acute oral toxicities of these chemicals for rats.

2. Human and rat leucocytes are equally susceptible to the primary toxic action of the alcohols, and the toxicity of the alcohols for these cells increases with molecular weight. This increase of toxicity with increase in molecular weight follows closely the geometric progression $1 : 3^1 : 3^2 : 3^3 : 3^4 : 3^5$ noted by Kamm.

3. The acute oral toxicity to rats of the first four primary alcohols increases with increase in molecular weight, while amyl, hexyl, and heptyl alcohols decrease in toxicity with increase in molecular weight.

4. Rat leucocytes are more susceptible than human leucocytes to the primary toxic action of acid and neutral solutions of acetic acid and the chloracetic acids. Acid solutions of the four acids were toxic for leucocytes in approximately equimolecular concentrations. Neutral solutions of the four acids were toxic for leucocytes in the same concentrations.

5. The acute oral toxicity to rats of acetic acid and the chloracetic acids bears no relation to molecular weight, since monochloracetic acid is approximately forty times more toxic than the other members of the series.

6. There is a marked difference between "primary" toxicity as measured by the direct action of a chemical on an isolated tissue cell and "functional"

toxicity as determined by the administration of the chemical where the toxic action is directed against the body as a whole.

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AN UNUSUAL TEMPERATURE COURSE IN INFECTIOUS MONONUCLEOSIS*

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SIR WILLIAM OSLER dubbed syphilis the great mimicker of other diseases. Infectious mononucleosis with its protean manifestations is fast becoming a successful runner-up to lues in this regard. Bernstein,[1] in his excellent review of infectious mononucleosis, demonstrates that this disease may affect almost all of the body systems. Because the recording of each new manifestation of this disease will lead to its complete understanding, the report of an unusual temperature curve is presented.

REPORT OF CASE

A white male aged 25 years was first seen on the sixth day of his illness. He complained of malaise, fever, and swelling of the glands of the left side of the neck. Physical examination revealed a temperature of 101° F. and enlarged glands of the left posterior cervical chain. Excepting for later involvement of the right posterior cervical and right occipital lymph glands, the physical examination was entirely negative throughout the course of the illness. On the twelfth, fourteenth, sixteenth, eighteenth, and twenty-second evenings of the illness (Fig. 1), the patient had a sudden chill, violent enough to shake the entire bed, followed by a rapid rise in the temperature to levels between 101° F. to 104.8° F. and profuse sweating. In each instance the temperature fell by crisis within a period of two

*Received for publication, January 18, 1943.

to three hours. The chill, which lasted from ten to twenty minutes, was always preceded by an ache in the lower back which increased in intensity as the temperature rose and subsided as the temperature fell. The white blood count on the eleventh day of the illness was 7,950 with 53 per cent neutrophils (38 per cent segmented; 15 per cent nonsegmented), 40 per cent lymphocytes, and 7 per cent monocytes. The heterophile antibody reaction was negative. Two smears for malarial parasites were negative. On the thirteenth day a blood culture was negative, and agglutination reactions for typhoid "O," typhoid "H," paratyphoid "A," paratyphoid "B," proteus X19 (typhus fever), and *Brucella abortus* were negative in all dilutions. On the fifteenth day of the illness, the heterophile antibody reaction was positive in a dilution of 1:64. The white blood count was 14,700 with 28 per cent neutrophils (24 per cent segmented; 4 per cent nonsegmented), 1 per cent eosinophiles, 51 per cent small lymphocytes, 18 per cent pathologic lymphocytes, and 2 per cent monocytes. The presence of these pathologic lymphocytes, which were described by Downey and McKinlay,[2] is characteristic of infectious mononucleosis. Several urinalyses were negative. After the chill and fever of the twenty-second day, the temperature fell to normal and remained there. The patient made an uneventful recovery.

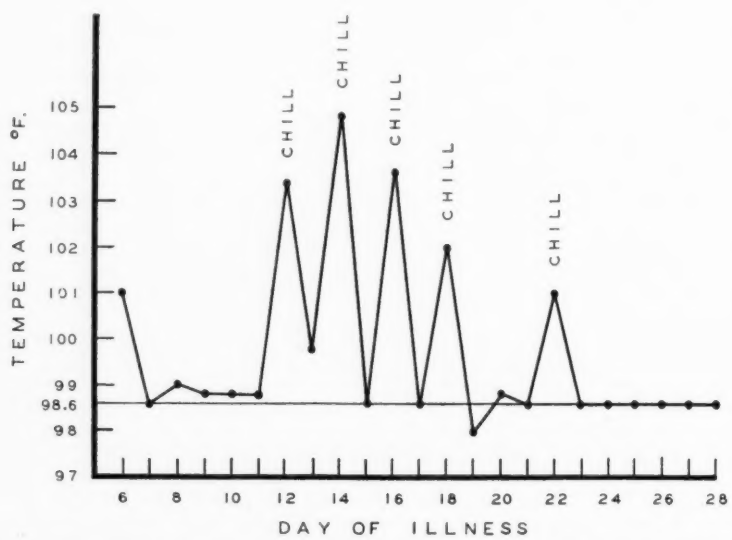

Fig. 1.—Each point represents the highest temperature for that particular day.

COMMENT

Bernstein,[1] in whose monograph on infectious mononucleosis the literature to 1940 is reviewed, does not mention such a temperature course. He further states[3] that in his personal experience he has never seen this disease behave in this way. Anyone who has dealt with the problems in the differential diagnosis of this disease may readily understand our difficulties until the true nature of the illness revealed itself. Our first clinical impressions included infectious mononucleosis, but the heterophile antibody reaction was still negative on the eleventh day. Then the series of chills and fever began. The possibility of malarial infestation was ruled out by two negative blood smears for the presence of the parasites. Sepsis and the enteric fevers were eliminated by the negative blood culture and agglutination tests. The lymphadenopathy and lymphocytosis brought to mind the possibilities of either an acute lymphatic leucemia or an acute Hodgkin's disease. The diagnosis was finally established by the positive

heterophile antibody reaction in a dilution of 1:64 and the presence of the abnormal lymphocytes[2, 4] in the blood smear. Whether or not suppuration or the breaking down of the retroperitoneal lymph glands was the cause of both the temperature response and the backache could only be surmised.

SUMMARY

A case of infectious mononucleosis with an unusual temperature course resembling that of malaria is presented. The absence of any previous mention in the literature of such a temperature response is noted.

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ATTEMPTS TO OBTAIN BETTER RESULTS WITH THE BACTERIAL ANTIGEN ("VACCINE") THERAPY OF LOW GRADE CHRONIC ("FOCAL") INFECTION

I. POSSIBLE ERRORS OF USUAL METHODS

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DURING the past seventeen years we and our associates have been making a careful study of the problem of low grade chronic ("focal") infection in an effort to determine its significance. Such a study included investigation of the field of bacterial antigen ("vaccine") therapy and has resulted in the development of diagnostic and therapeutic bacteriologic methods which we believe are superior to others we have tried. This series of reports presents a discussion of the progress achieved so far.

Although most physicians believe that there is little merit in the "vaccine" therapy of low grade chronic infections, its continued use by some suggests that it must have value. Differences of opinion as to its value might be based on differences in the technique of preparing and administering the "vaccines." That there may be room for improvement in manufacture, even in standardized vaccines that have been used extensively for a long time, is indicated by the re-

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With the cooperation and advice of Drs. Earl H. Eaton, Anthony A. Ressa, H. F. Rubenstein, and Edward C. Titus.

Aided by grants from the Ophthalmological Foundation, Inc.

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Received for publication, January 26, 1943.

cent radical change in the type of organism used in the preparation of typhoid vaccine.[1] Our findings indicate that the failure to secure results in bacterial antigen therapy of chronic low grade infections may be caused by: selecting cultures that are incapable of inciting the production of suitable antibodies or of suitable quantities of them; improperly treating the cultures; failing to provide adequate surgical and supportive measures; and using unsatisfactory dosage with respect to both the interval and dose. More specifically, the following factors may have a profound effect on the course of such treatment.

POSSIBLE BACTERIOLOGIC ERRORS

a. *Culture of unsuitable foci.*—In the search for possible foci of infection a significant focus may be overlooked and cultures may be made of other foci. For example, in a patient with chronic prostatitis and rheumatism the prostate was considered the obvious focus, but only nonpathogenic organisms were found in the prostatic fluid. Highly pathogenic organisms were found in the nasal and oral cavities, which were the seat of long-standing but almost asymptomatic infection. Quite often special attention is paid to one particular focus, while others may be ignored.

If, as seems reasonable on the basis of available data,[2] "*Streptococcus viridans*" is the common denominator of low grade chronic infections, then knowledge of the incidence of pathogenic types of this organism should prove useful in indicating their most likely sources. Ruggier and Chapman[3] found resistant (i.e., probably pathogenic) streptococci to comprise 72 per cent of streptococci from periapical pockets, 54 per cent of those from extracted teeth, 44 per cent of those from the pharynx, 35 per cent of those from the nasopharynx, and 28 per cent of those found in the feces ("enterococci" being ignored). By use of an improved technique pathogenic types of streptococci were found in the pharynx in *every* person with low grade infection. For this reason the pharynx (and any periapical pockets or extracted teeth), should be cultured in every case. (The most common source of pathogenic staphylococci is the nasal cavity, which likewise should be cultured routinely.) Additional cultures should be taken from other sources whenever there is suspicion of active infection. For example, in one patient there were only 200,000 pathogenic type streptococci (18 per cent of the total streptococci in the swab) in a swab from the pharynx, but there were 5,000,000 (36 per cent of those in the gingival swab) in a swab from the gum margins, many of which showed evidence of inflammation. (See also Ruggier and Chapman.[3])

b. *Use of improper cultural methods.*—Laboratories that are infrequently called upon to make cultural studies of focal infection have difficulty in maintaining a supply of suitable culture media. Hence some of them use the Loeffler medium slants, supplied by departments of health, which are frequently dry and unsatisfactory for this type of work. Others use "blood agar" plates of varying composition, poured so thin, or which are so old, that they are dry and unsuitable for adequate growth of pathogenic bacteria, many of which require abundant moisture. Usually the material is spread on only one plate, resulting in a crowded growth that makes the study of individual colonies difficult.

Unless appropriate cultural methods are used, streptococci, particularly anaerobic types, pneumococci, some members of the Hemophilus group, and other

fastidious organisms may not be recovered. Much tedious work can be eliminated by using the newer selective isolation methods. Penicillin, recently discussed by Chain et al.,[4] and Abraham et al.,[5] is useful in the selective isolation of the Hemophilus group. Bromthymol blue lactose agar and phenol red mannitol agar[6, 7] give superior results in the selective isolation of probable pathogenic staphylococci. Crystal violet, sodium azide, tryptose blood agar[8, 23] is of considerable assistance in isolating pathogenic streptococci from mixed cultures.

c. *Taxonomic errors and unsuitable taxonomic considerations.*—Often an organism diagnosed as "hemolytic streptococcus" may be a hemolytic member of the Hemophilus group, a hemolytic staphylococcus, or even an α- or γ-hemolytic streptococcus. The presence of a small hemolytic colony on the *surface* of "blood agar" does not warrant a diagnosis of "hemolytic streptococcus." Similarly, a mucoid colony of staphylococci or of streptococci is sometimes mistaken for *Klebsiella pneumoniae*. Organisms received as "staphylococci" were found to be "enterococci" and *Neisseria catarrhalis*, and a "Type IV pneumococcus" proved to be *Streptococcus salivarius*.

Sometimes an organism is designated by an insignificant property. For example, numerous publications refer to "hemolytic" staphylococci but it has been shown[9] that the power of staphylococci to produce hemolytic zones on blood agar has only secondary significance.

d. *Error of random selection of colonies.*—When colonies are selected at random from a mixed culture they may not be representative of apparently similar colonies elsewhere on the plate. Only rarely in this type of work do we deal with cultures in which all the cells have similar properties. Therefore, in selecting a colony at random, even from an apparently pure culture, it is possible to select one that does not represent the entire culture.

e. *Influence of the age of the culture.*—Most bacteriologists harvest a culture after "overnight" incubation, an interval which may include extremes of sixteen and thirty hours. Such differences in age are often accompanied by differences in properties of the cultures.[10, 11] (We have found that late logarithmic cultures make more efficient antigens.) Metabolites, particularly hydrogen ions, may have an unfavorable effect on bacteria and their antigens. Polysaccharides, which may be related to the specificity of a bacterial antigen, are not produced uniformly throughout the period of growth of the culture. Autolytic products, the result of prolonged incubation, may alter the antigenic complex. For these reasons, it is important to know the influence of age on the antigenic structure of different cultures.

f. *Fallacy of considering the predominant organism as the focal invader.*—Many bacteriologists in medical fields consider only the gross features of a culture and make no more than a casual inspection of the plates. The relative number of the different types of organisms is estimated on the assumption that the organisms present in largest numbers are most likely to be the focal invaders. While this is often true, the rich growth of saprophytes in many parts of the body precludes the possibility of recognizing focal bacteria on the basis of predominance.

g. *Inadequacy of methods used to differentiate etiologic bacteria from contaminants.*—Because cultures from suspected foci often contain bacteria which

have no relationship to the infection, it is important that attempts should be made to differentiate these contaminants from the organisms causing the infection.

Intradermal tests, complement fixation reactions, agglutination reactions, and qualitative bacteriostatic tests have been shown to be inadequate for such differentiation.[12, 13] There is no evidence that organisms showing specific localization produce better antigens than do those not localizing in particular tissues.

Our researches[13] indicate that differentiation between causative organisms and contaminants can be made by methods based on the probability that bacteria causing infection are more likely than are contaminants to possess properties associated with pathogenicity. (It is possible that strains of low pathogenicity may be responsible for infections in tissues having low resistance; but such instances are uncommon and only a mild infection usually results.) Because of the difficulty of applying animal inoculation tests, studies were made of in vitro properties. It was found that certain of these properties were parallel with pathogenicity for animals,[9, 14, 15] were parallel with other findings[2, 9, 16, 17, 23] in the patients from whom they had been isolated, and, because they were easily reproducible, were suitable for studying large numbers of cultures.

h. *Selection of a nonpathogenic variant.*—Even though accurate methods are used to differentiate pathogenic from nonpathogenic strains, the culture selected may still be nonpathogenic because pathogenic cultures often contain nonpathogenic variants. If different colonies are tested separately, an apparently pure culture will be found in many cases to consist of a mixture of dissociants (or variants) in which different properties may be distributed in different patterns among the different cells of the culture.[3] Because this phenomenon occurs so frequently in α- and γ-hemolytic streptococci, cultures of these organisms should be considered, not as stable homogeneous masses, but as dynamic mixtures of cells with a strong tendency to instability of the biochemical, physiologic, immunologic, and pathogenic properties of the different cells of the cultures. To be fully representative, a culture must not only be genetically related to the etiologic (pathogenic) culture but must also possess its pathogenic properties.

Not much attention appears to have been paid to the possibility that the antigenic structure of a culture may change along with the dissociation of other characters. If the colony or colonies selected for preparing the growth for the antigen should lack any of the antigenic properties of the parent culture, then the antibodies produced from injection of this antigen may be qualitatively and quantitatively different from those necessary to neutralize the antigenic complex produced by the invading microorganisms.

LACK OF SPECIFICITY OF THE ANTIGEN

Even though considerable care is exercised in selecting the culture, there is still a possibility that the antigen prepared from it may not be efficient for the following reasons:

a. *There may be loss or degeneration of the antigenic properties during cultivation of the microorganism.*—Any degeneration of the culture during growth is likely to be associated with degeneration of the antigenic complex. Therefore,

steps should be taken to minimize degeneration by preventing excessive variations in hydrogen-ion concentration and excessive oxygenation, and by harvesting the culture as quickly as possible.

b. *Physical or chemical changes may be produced in the antigen after the culture has been harvested.*—Heat so injures many bacterial antigens that it is necessary to inject several million bacteria to produce a physiologic effect. In our experience 0.5 per cent phenol does not impair the immunogenic properties of staphylococci, streptococci, and Klebsiella, and does not appear to be injurious to the patients in the dosages used. Only rarely do we find sensitivity to phenol. With properly prepared antigens a physiologic effect may be produced with amounts representing less than a single bacterial cell. The antigens should be stored in resistant glass bottles to minimize the accumulation of alkali.

The essential property of a bacterial antigen is that it should be an efficient antibody-stimulating preparation. If the concentration of specific antigen should be too small (as in heated or nonspecific vaccines), the dose required to provoke a high titer of antibodies will contain so much protein that the protein itself may produce local or even general reactions.

UNSUITABLE METHODS OF VACCINE ADMINISTRATION

a. *Fallacy of attempts to force the patient to produce a high titer of antibodies.*—The usual aim in vaccine therapy is to force the patient to produce large amounts of antibodies. For this reason most schedules call for the administration of progressively larger doses to the limit of tolerance, even though the immediate results should be unfavorable. Not only is an unfavorable effect ignored, but quite often it is considered a favorable sign, an indication that the vaccine is having an effect on the patient.

On the contrary, such an unfavorable effect, if more than transitory, usually indicates that the patient is unable to produce a sufficient quantity of antibodies to neutralize the antigen. Consequently the excess antigen acts as a toxin. Recovery might be possible if sufficient opportunity were given for the tissues to produce more antibodies, but often the patient is made worse by another injection before he has had a chance to recover. When an injection is given too soon after the previous one it augments the residual antigen, resulting in even greater toxicity.

b. *Use of empirical methods for determining dosage.*—Because the laws governing response to the introduction of an antigen into the body are poorly understood and because of the difficulty in determining the precise effects, it is customary in administering antigens to follow a fixed schedule such as is suggested by manufacturers of biologic products. While such a procedure works satisfactorily in the prophylactic immunization of healthy persons (e.g., against typhoid), it is not reasonable to expect that it would work equally well in immunizing body tissues against an organism with which they are already infected. The varying amounts of antigen produced by the pathogenic organisms present, the varying damage done to tissues by these organisms, as well as the varying effects of nutritional and endocrine deficiencies, combine to alter the ability of the body to respond to the introduction of additional antigen. In our experience best results have been obtained by adjusting the dosage (i.e., both the dose and

the interval) according to the response to each injection of antigen. This subject, including our methods of estimating response to antigen injections, will be discussed in a later paper.

c. *Improper spacing of injections.*—The response to an injection of antigen is a function of the properties of the antigen, which are constant, and of the tissue reactability and antibody concentration, which vary with the interval. Therefore, it is important to space the injections to take advantage of these variations. If an antigen injection is given too soon after a previous injection, enough unneutralized antigen may still remain in the tissues to make the amount being injected too large. If given too late, the antibody level may have dropped to such a low level that several injections may be required to overcome this slump and obtain optimum response.

d. *Errors in the size of the dose of antigen.*—An inadequate dose of antigen will have only weak power of stimulating the production of antibodies. The closer a dose is to the optimum, the greater will be the degree of stimulation. However, maximum stimulation comes only from a quantity of antigen which is close to an overdose. Consequently, considerable care must be exercised when a particular dose produces marked stimulation, because a slightly higher dose, or the same dose repeated too soon, may cause unfavorable symptoms.

At times, following the administration of a dose which is considerably too large, the signs of overdosage may not be dramatic and may be mistaken for failure to respond to the antigen injection. Even if such a dose is repeated several times, the evidence of overdosage may be difficult to detect. We have seen a number of patients, usually of low vitality, who showed no evident change, either beneficial or otherwise, during a series of antigen injections, but who improved after an interval of several weeks without injections. Later these patients responded satisfactorily to smaller doses of antigen.

During an acute illness, or shortly after surgical removal or treatment of foci of infection, there is often so much circulating toxin that even minute amounts of antigen act as overdoses. Consequently, great care must be taken in giving injections in such cases. In general, it is advisable to postpone antigen therapy until a week after any acute illness has subsided, or until the immediate unfavorable effects of a surgical procedure have disappeared and improvement is evident.

In the early stages of treatment favorable response to the injection of antigen follows a curve: increasing improvement, followed by gradual return to the basic level. An injection of antigen will produce better results if it is given at the end of the period of improvement rather than after complete return to the basic level. When the immunity has been raised to a satisfactory level, the antigen may be given in rapidly increasing doses at frequent intervals.

INADEQUATE SUPPORTIVE MEASURES

a. *Failure to reduce the toxic load.*—Response to the injection of an antigen depends not only on considerations of the antigen but also on tissue reactability, which in turn is affected by the toxic load on the system. An undrained (i.e., confined) focus of infection is a source of toxic material. When the focus is

drained or removed much of this is eliminated, lightening the toxic load on the body. This is one of the most important phases of bacterial antigen therapy. In many instances in which the patient is sensitive to extremely small doses of antigen it will be found that there is an untreated focus. Numerous patients who reacted badly to small doses of antigen had a beneficial response to much larger doses of the same antigen after foci of infection had been drained or removed.

The toxic load may also be reduced by appropriate chemotherapy and physiotherapy.

b. *Failure to correct endocrine dysfunction.*—A lowered basal metabolic rate has been reported as a common finding in chronic arthritis[18] and in chronic low grade infections.[19] In patients who become drowsy after a dose of antigen that is higher than the optimum amount, the drowsiness may often be reduced by giving adequate amounts of thyroid preparations simultaneously with the vaccine injection. In patients with more definite evidence of thyroid hypofunction the continued use of thyroid preparations may prove markedly beneficial. Often, in these cases, thyroid function improves as the general health improves, and thyroid therapy may eventually be discontinued.[20]

Sex gland and adrenal deficiencies may also require attention.

c. *Failure to correct liver dysfunction.*—Liver dysfunction is common in arthritis[21] and other forms of low grade chronic illness.[22] Dietary adjustment (low fat and cholesterol, high carbohydrate and high protein) and the administration of bile salts are useful in correcting liver disturbances and in improving the response to vaccine therapy.

d. *Failure to supply adequate nutrition.*—It is generally recognized that resistance to infection is dependent to a certain extent upon nutritional factors. Dramatic response is sometimes noted when an adequate diet is supplied, or even when a single specific dietary factor is administered. The beneficial effects of the appropriate use of vitamin B complex have been especially noteworthy.

With careful attention to the details enumerated and by application of the principles to be discussed in the following papers, we believe it possible to obtain better results in the bacteriologic investigation and treatment of low grade chronic infections than are possible by other methods that have been proposed for this purpose.

SUMMARY

An analysis of some of the factors that might prevent optimum response to the bacterial antigen ("vaccine") therapy of low grade chronic ("focal") infections reveals the following possibilities:

The cultures may be taken from unsuitable foci. The recovery of organisms of suitable antigenicity may not always coincide with clinical expectations.

The cultural procedure may be inadequate from the standpoint of the technique of isolation, the method of selecting the colonies, the incubation period, taxonomic considerations, and the methods used to differentiate the etiologic bacteria from contaminants.

The culture selected may not possess suitable antigenic properties. The antigen may be injured during the cultivation process or in the later manipula-

tion. The injured antigen may contain an excessive ratio of protein to specific antigen, causing it to be toxic and nonspecific.

The dosage may be unsuitable. The injections may be given too often or the amounts may be too large. In other instances the doses may be too small for adequate stimulation of antibodies. The use of a fixed schedule is condemned.

The condition of the patient may not be favorable for optimum antibody response because of endocrine or nutritional deficiencies, liver dysfunction, an excessive toxic load (e.g., an untreated focus), or other factors.

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TREATMENT OF EXPERIMENTALLY INDUCED TYPE I PNEUMOCOCCUS PNEUMONIA IN ALBINO RATS*

A Comparative Study of the Therapeutic Efficiency of Various Sulfonamides and Specific Rabbit Antipneumococcus Serum and Combinations of the Two

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GROSS and Cooper,[1] in 1937, treated Type I pneumococcal infections in rats with sulfanilamide and rabbit antipneumococcal serum. They found that sulfanilamide was better than serum, and that when the two were combined they were more effective than either therapy alone.

In 1939, Kepl and Gunn[2] similarly treated Type I pneumococcal infections in rats with sulfapyridine and specific rabbit serum. They concluded that when serum was begun within four hours after inoculation, the combined use of serum and sulfapyridine proved more effectual than when either was used alone. When infection was well established, however, serum was more efficacious than sulfapyridine, and at this stage the combination of serum and sulfapyridine produced results no better than when serum was given alone.

In 1940, Wright and Gunn[3] reported on the treatment of experimental Type III lobar pneumonia in rats with sulfapyridine and serum. Their results differed somewhat from those of Kepl and Gunn. Wright and Gunn found that, when the infecting dose of Type III pneumococci was relatively small, serum and sulfapyridine were equally efficient, but that when the infecting dose was large, producing a mortality rate of 100 per cent in control animals, the mortality rates with sulfapyridine were lower than those obtained with serum. A combination of serum and sulfapyridine did not reduce the mortality below that obtained with sulfapyridine alone.

In each of these studies the animals were inoculated intrabronchially with pneumococci suspended in gastric mucin. None, however, report on blood cultures prior to and during treatment of the animals, making it difficult therefore to evaluate accurately the effectiveness of the therapy used, except from a standpoint of gross mortality rates. The lack of reports on the results of blood

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Received for publication, February 1, 1943.
The sodium sulfathiazole used in this study was donated by the Winthrop Chemical Company. The dimethyl sulfadiazine used in this study was donated by the Nepera Chemical Company.

cultures makes it impossible to determine how rapidly the therapy was able to dispose of the bacteremic state in those animals which survived.

In 1941,[4] we reported a study of the comparative efficacy of sulfapyridine and rabbit serum when used alone and in combination in treating artificially induced Type I pneumococcal infections in albino rats. We found that in these infections, which, during the animal's life, were manifested by positive blood cultures, type specific serum appeared to be more potent than sulfapyridine alone; whereas, a combination of sulfapyridine and serum, at approximately one-half the dose of either used separately, gave results similar to those obtained with serum alone.

The present report concerns a similar study made to determine the comparative effectiveness of other sulfonamide drugs when used alone or combined with specific antipneumococcal serum in the treatment of Type I pneumococcal infection in albino rats. The sulfonamide drugs employed were sulfathiazole, sodium sulfathiazole, sulfadiazine, sodium sulfadiazine, and dimethyl sulfadiazine. The last-mentioned drug, although used effectively in the treatment of pneumococcal pneumonia in human beings during the past year in England under the name of sulfamethazine,[5] is new to the field of chemotherapy in this country.

METHODS

Inoculation of the rats was accomplished by spraying small amounts of a pneumococcal culture into both the pharynx and nares, a method described in a previous paper.[6] The culture, which was suspended in 15 c.c. of a physiologic solution of sodium chloride, consisted of the washings of the peritoneal cavity of a white mouse infected with Type I pneumococci (R1 strain), and contained, as a result of the peritoneal inflammation, natural mucin in addition to the other components of an inflammatory exudate. The procedure used to inoculate the animals was entirely nontraumatizing, and, we believe, simulated the manner in which human pneumococcal infections occur.

A total of 137 rats, weighing approximately 300 grams each, were thus inoculated. Of these, 125 animals (91 per cent) became infected as manifested by blood cultures positive for Type I pneumococci. Only those animals developing positive blood cultures were used in this study.

Cultures of the blood were made just prior to the initial treatment, at 24 hours after inoculation, and at 48 hours, and 72 hours, just prior to succeeding treatments. They were then made at daily intervals until death ensued or until no growth was obtained. The blood for culture was plated on Avery's medium for pneumococci, to which was added 5.0 mg. per 100 c.c. of paraminobenzoic acid.

All the animals used, whether dying spontaneously before the fifteenth day or sacrificed on the fifteenth day, were autopsied.

Although 125 animals were successfully infected, 114 were included in this report, since in 11 animals the suspensions or solutions of the sulfonamide drugs were introduced accidentally into the trachea, producing immediate death. There was, therefore, an unequal distribution of animals in some of the groups.

To insure accurate dosage, the sulfonamide compounds, either suspended or dissolved in distilled water, were administered by gavage. The antipneumococcal serum was administered intraperitoneally by injection.

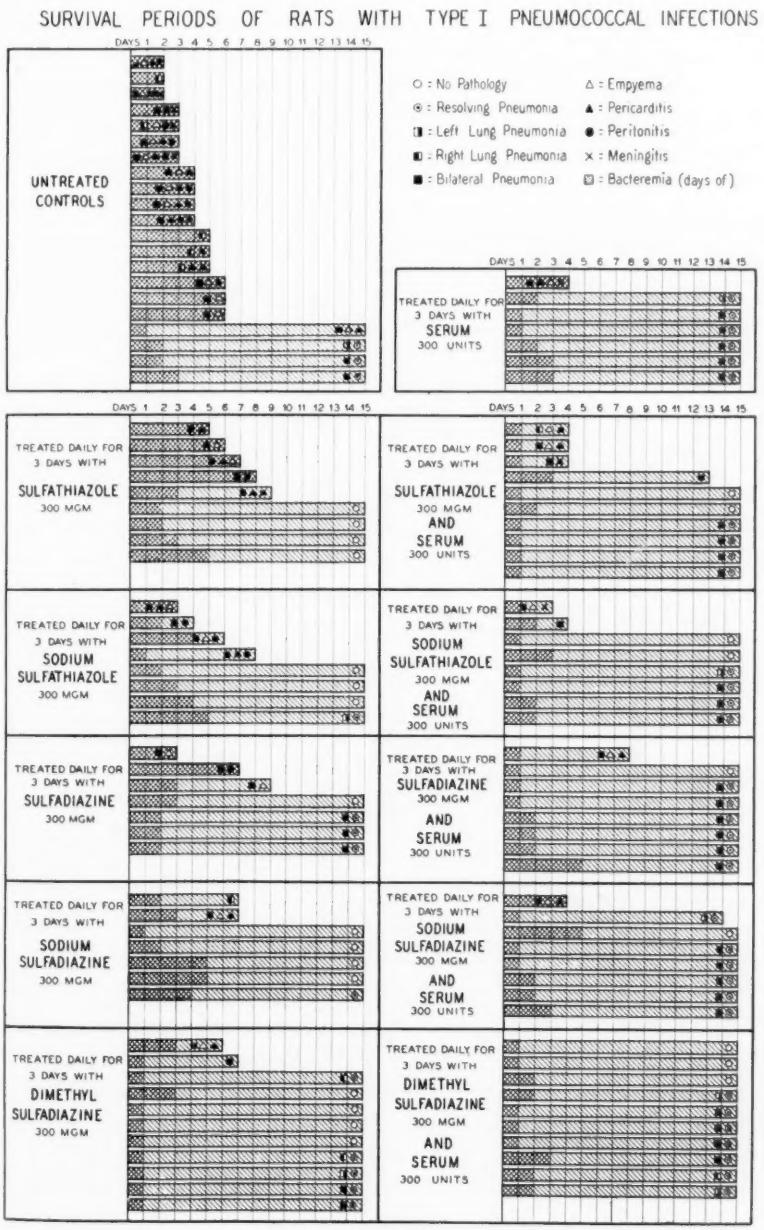
SURVIVAL PERIODS OF RATS WITH TYPE I PNEUMOCOCCAL INFECTIONS
DAYS 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
UNTREATED CONTROLS
○ : No Pathology
◉ : Resolving Pneumonia
◨ : Left Lung Pneumonia
◧ : Right Lung Pneumonia
■ : Bilateral Pneumonia
△ : Empyema
▲ : Pericarditis
● : Peritonitis
× : Meningitis
▨ : Bacteremia (days of)
TREATED DAILY FOR 3 DAYS WITH SERUM 300 UNITS
TREATED DAILY FOR 3 DAYS WITH SULFATHIAZOLE 300 MGM
TREATED DAILY FOR 3 DAYS WITH SULFATHIAZOLE 300 MGM AND SERUM 300 UNITS
TREATED DAILY FOR 3 DAYS WITH SODIUM SULFATHIAZOLE 300 MGM
TREATED DAILY FOR 3 DAYS WITH SODIUM SULFATHIAZOLE 300 MGM AND SERUM 300 UNITS
TREATED DAILY FOR 3 DAYS WITH SULFADIAZINE 300 MGM
TREATED DAILY FOR 3 DAYS WITH SULFADIAZINE 300 MGM AND SERUM 300 UNITS
TREATED DAILY FOR 3 DAYS WITH SODIUM SULFADIAZINE 300 MGM
TREATED DAILY FOR 3 DAYS WITH SODIUM SULFADIAZINE 300 MGM AND SERUM 300 UNITS
TREATED DAILY FOR 3 DAYS WITH DIMETHYL SULFADIAZINE 300 MGM
TREATED DAILY FOR 3 DAYS WITH DIMETHYL SULFADIAZINE 300 MGM AND SERUM 300 UNITS

Chart I.

DOSAGE OF RABBIT ANTIPNEUMOCOCCAL SERUM AND THE SULFONAMIDES

A series of 21 animals, in which no therapy had been given, was used as a control for those receiving treatment. Therapy with serum, sulfonamides, and

combinations of the two was begun at twenty-four hours after inoculation with pneumococci.

Serum.—Three hundred units of concentrated Type I rabbit antipneumococcal serum were given on three successive days to each animal of a second series.

Sulfonamides Alone.—To a third series, 300 mg. of a sulfonamide drug per animal (sulfathiazole, sodium sulfathiazole, sulfadiazine, sodium sulfadiazine, or dimethyl sulfadiazine) was given alone to each of five groups of animals on three successive days.

Sulfonamide and Serum Combined.—A combination of 300 mg. of one of the sulfonamides and 300 units of antipneumococcal serum per animal was administered simultaneously daily for three days to each of five groups of rats of a fourth series.

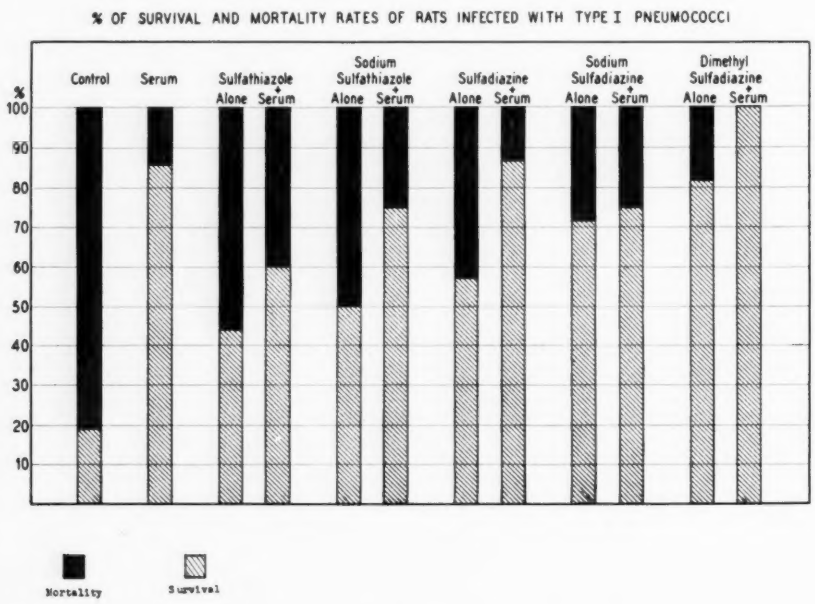

Chart II.

RESULTS

Series I, Control Animals.—Seventeen of the twenty-one rats in this series died. The mortality rate was 81 per cent. The average period of survival of those rats dying spontaneously was 3.9 days. Of these, two had pneumonia alone. Three animals had, in addition to pneumonia, one complication; four had two complications; six had three; and two had four complications. Of the four animals surviving fifteen days and sacrificed, three had resolving pneumonia, while one had bilateral pneumonia complicated by empyema and pericarditis. The average duration of bacteremia of the surviving animals was two days.

Series II, Serum Alone.—One of the seven animals in this series died, giving a mortality rate of 14.3 per cent. This rat, which died spontaneously on the fourth day, had bilateral pneumonia, complicated by empyema, pericarditis,

TABLE I

	NUMBER OF RATS			MORTALITY RATE	AVER. PERIOD OF SURVIVAL OF THOSE DYING SPONTANEOUSLY	AVER. DURATION OF BACTEREMIA OF SACRIFICED RATS
	TOTAL	SURVIVING 15 DAYS	SPONTANEOUS DEATHS	PER CENT	DAYS	DAYS
Series I—Controls	21	4	17	81.0	3.9	2.0
Series II—Serum alone	7	6	1	14.3	4.0	2.0
Series III—Chemotherapy alone						
Group "A"—Sulfathiazole	9	4	5	55.6	7.0	3.0
Group "B"—Sodium sulfathiazole	8	4	4	50.0	5.3	3.5
Group "C"—Sulfadiazine	7	4	3	42.9	6.3	2.3
Group "D"—Sodium sulfadiazine	7	5	2	28.6	9.0	3.4
Group "E"—Dimethyl sulfadiazine	11	9	2	18.2	4.5	1.2
Series IV—Sulfonamide and serum						
Group "A"—Sulfathiazole and serum	10	6	4	40.0	5.5	1.2
Group "B"—Sodium sulfathiazole and serum	8	6	2	25.0	3.5	1.7
Group "C"—Sulfadiazine and serum	8	7	1	12.5	8.0	2.0
Group "D"—Sodium sulfadiazine and serum	8	6	2	25.0	9.0	2.3
Group "E"—Dimethyl sulfadiazine and serum	10	10	0	0		1.6

and meningitis. All those sacrificed had resolving pneumonic processes. The average duration of bacteremia of those sacrificed was two days.

Series III, Chemotherapy Alone.—Group "A" Sulfathiazole.—Five of nine rats died. The mortality rate was 55.6 per cent. The average period of survival of those dying spontaneously was seven days. Three had but one complication; while two had two complications. The four animals which survived for fifteen days had no pathology at autopsy. The average duration of bacteremia was three days in the sacrificed animals.

Group "B" Sodium Sulfathiazole.—Four of eight rats in this group died, the mortality rate being 50 per cent. The average period of survival of those dying spontaneously was 5.3 days. Two had pneumonia with one complication and two had two complications. Three of those sacrificed at fifteen days had no pathology, while one had resolving pneumonia. The average duration of bacteremia of the sacrificed animals was 3.5 days.

Group "C" Sulfadiazine.—Three of seven rats died. The mortality rate was 42.9 per cent. The average period of survival of those dying spontaneously was 6.3 days. Each of these animals had but one complication in addition to pneumonia. Of the four animals sacrificed on the fifteenth day, one had no pathology, while three had resolving pneumonia. The average duration of bacteremia of the sacrificed rats was 2.25 days.

Group "D" Sodium Sulfadiazine.—There were seven animals in this group. Two died; each on the seventh day, giving a mortality rate of 28.6 per cent. One had pneumonia alone; the other had pneumonia complicated by empyema and pericarditis. Four of the five animals sacrificed on the fifteenth day had no

pathology, while one had resolving pneumonia. The average duration of bacteremia of those sacrificed was 3.4 days.

Group "E" Dimethyl Sulfadiazine.—Of 11 animals treated, two died, one on the sixth and one on the seventh day, giving a mortality rate of 18.2 per cent; these had respectively bilateral pneumonia, empyema, and pericarditis; and bilateral pneumonia. Of the nine animals which were sacrificed on the fifteenth day, four had no pathology and five had resolving pneumonia. The average duration of bacteremia of the sacrificed animals was 1.22 days.

Series IV, Sulfonamide and Serum Combined.—Group "A" Sulfathiazole and Serum.—There were ten rats in this series. Four, or 40 per cent, died, three on the fourth day and one on the thirteenth day. The average period of survival was 5.5 days. The latter had bilateral pneumonia alone; of the other three, two had pneumonia, empyema, and pericarditis, and one had pneumonia and meningitis. Four of the six rats sacrificed on the fifteenth day had resolving pneumonia, and two had no pathology. The average duration of bacteremia of the sacrificed animals was 1.17 days.

Group "B" Sodium Sulfathiazole and Serum.—Two (25 per cent) of eight rats in this series died on the third and fourth days respectively after inoculation. The former had bilateral pneumonia, empyema, and meningitis; the latter, bilateral pneumonia. The mortality rate was 25 per cent. Of the six animals surviving fifteen days and sacrificed, two had no pathology and four had resolving pneumonia. The average duration of bacteremia of those sacrificed was 1.7 days.

Group "C" Sulfadiazine and Serum.—One of eight rats in this group died, giving a mortality rate of 12.5 per cent. This animal, which died on the eighth day, had bacteremia for one day, and at autopsy, had bilateral pneumonia, empyema, and pericarditis. The seven animals sacrificed on the fifteenth day, with one exception having no pathology, had resolving pneumonia. The average duration of bacteremia of those sacrificed was two days.

Group "D" Sodium Sulfadiazine and Serum.—Two of eight rats in this group died, the mortality rate being 25 per cent. These animals died on the fourth and fourteenth days and respectively had pneumonia, empyema, and pericarditis; and resolving pneumonia. One had no pathology. The average duration of bacteremia of those sacrificed was 2.33 days.

Group "E" Dimethyl Sulfadiazine and Serum.—Ten rats were treated and none died. All ten rats were sacrificed on the fifteenth day. Seven at autopsy had resolving pneumonia, while three had no pathology. The average duration of bacteremia was 1.6 days.

There were no instances of toxicity resulting from any of the therapeutic substances used.

SUMMARY AND CONCLUSIONS

1. Type I pneumococcal infection as manifested by positive blood cultures was produced in 114 albino rats. In those rats dying spontaneously, pneumonia, and in most instances such complications as empyema, pericarditis, peritonitis, or meningitis, were found at post-mortem examination.

2. Sulfathiazole, sodium sulfathiazole, sulfadiazine, sodium sulfadiazine, dimethyl sulfadiazine, and concentrated refined Type I rabbit antipneumococcal serum were used alone and in combination and administered to 93 animals, treatment being started at twenty-four hours after inoculation.

3. In Type I pneumonia in rats, treatment with serum alone and dimethyl sulfadiazine alone resulted in higher survival rates than with any of the other sulfonamides when used alone; whereas, a combination of serum and the sulfonamides gave better results in all instances than the respective sulfonamide used alone. Dimethyl sulfadiazine in combination with Type I serum resulted in higher survival rates than did any of the other sulfonamides. Sulfadiazine combined with serum appeared to be next in effectiveness, while sodium sulfathiazole, sodium sulfadiazine, and sulfathiazole, each combined with serum, were less effectual in this order in saving animals.

When compared with dimethyl sulfadiazine alone, sodium sulfadiazine, sulfadiazine, sodium sulfathiazole, and sulfathiazole were less effective in this order in producing survivals.

4. Sulfathiazole and sulfadiazine were more efficacious in disposing of bacteremia than were their respective sodium salts.

5. Dimethyl sulfadiazine, when used alone, cleared the blood stream of bacteremia more rapidly than any of the other sulfonamides alone, serum alone, or the sulfonamides combined with serum.

We wish to express our appreciation to Doctors Tasker Howard, Wade Oliver, and Arnold Eggerth for their careful criticisms and suggestions in this study.

We wish to thank also Mr. Sidney M. Karlton for his cooperation.

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CLINICAL CHEMISTRY

SIMULTANEOUS PERFORMANCE OF WELTMANN SERUM COAGULATION TEST, CEPHALIN FLOCCULATION TEST, AND MODIFIED TAKATA-ARA REACTION AS AN AID IN THE DIFFERENTIAL DIAGNOSIS OF LIVER DISEASE*

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AMONG the many procedures devised for the correct diagnosis of liver diseases, those which can be performed without too much inconvenience to the patient and which can be done without too much technical difficulty are of definite advantage. Takata-Ara reaction, cephalin flocculation test, and Weltmann serum coagulation test can be performed in one serum sample in a fairly simple way. It is the intention of this paper to show that the simultaneous use of all three tests may be helpful in arriving at a correct differential diagnosis in a patient suffering from disease of the liver.

The Weltmann serum coagulation reaction was performed according to the original paper of Weltmann.[1, 2] The stock solution of 5 per cent calcium chloride is best prepared by dissolving 6.7 Gm. calcium chloride .2 H_2O in 100 c.c. distilled water. The final solutions which are prepared from this stock solution are numbered from one to ten. A solution 7½, containing 0.35 c.c. stock solution in 100 c.c. water, is also used. A clearly recognizable coagulation appears in the first 6 or 7 test tubes in sera from normal individuals after the test tubes containing the final dilutions of $CaCl_2$ and serum have been boiled for fifteen minutes. Under abnormal conditions, coagulation may occur in fewer than 6 test tubes, and the coagulation band, as it is called by Weltmann, is shortened or shifted to the left. Or there is coagulation in more than 7 test tubes, and the coagulation band is lengthened or shifted to the right.

Takata-Ara Reaction.—The Takata-Ara reaction was performed in the modification of Mancke and Sommer.[4] By using this method the amount of serum is kept constant, but the concentration of mercuric chloride is varied. The advantage of using this modification is the possibility of grading the reaction in a fairly simple way. No flocculation or flocculation up to the second tube constitutes a negative reaction. Flocculation up to the third or fourth tube is considered a weak positive, and flocculation beyond the fourth tube is considered a strong positive reaction.

Cephalin Flocculation Test.—The cephalin test[4, 5] was performed with the commercially obtainable Difco product. According to Mateer and co-workers,[6] this product, which is made of an especially ripened cephalin, gives reliable results. Doubtful reactions as well as weak positive ones were not regarded as positive.

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Received for publication, May 28, 1943.

Fifty-two cases of primary liver disease or presenting jaundice and 70 control cases were used. The liver cases were divided into three groups: cirrhosis of the liver, diffuse hepatitis, and obstructing jaundice. Only those cases were used in which the diagnosis was reasonably certain. Cases not suffering from liver disease were used as controls.

Results.—(a) Diffuse Hepatitis.—In one of the 13 cases of diffuse hepatitis the Weltmann test showed a slight shortening of the coagulation band, in three cases a slight prolongation, and in 9 cases a marked prolongation. It is noteworthy that the most pronounced shift to the right of the coagulation band was observed in this group. (In 3 cases coagulation included dilution 9.) The Takata-Ara reaction was marked positive in 3 cases, slightly positive in one case, and negative in the remaining 9 cases. The cephalin test was positive in 7 out of 13 instances.

(b) Obstructive Jaundice.—Twenty-six cases of obstructive jaundice were examined. In 12 cases the obstruction was due to neoplasm, in 14 cases to stones. In the cases in which the obstruction was due to neoplasm, the coagulation band was in 3 cases shortened, in seven cases normal, and in only 2 cases slightly prolonged (dilution 7½). In the cases of obstructive jaundice due to stones, the Weltmann test showed in four cases a shortening and in 10 cases a normal range. The Takata-Ara reaction was one time strongly and 3 times weakly positive in the cases of obstructive jaundice due to stones; twice strongly and twice weakly positive in the neoplastic group. The cephalin test was three times positive in the stone group and twice positive in the other group, altogether 5 times in 26 cases. Erroneous results of one of the tests are frequently counterbalanced by the correcting results of the other tests. For instance, one case in this group showed a positive cephalin test, but the Takata-Ara reaction was negative and the coagulation band was shortened to 2.

(c) Cirrhosis.—In 14 cases of cirrhosis, 8 showed a marked and 2 a moderate prolongation of the coagulation band. In four cases the coagulation bands were normal. The Takata-Ara reaction was strongly positive in twelve cases, weakly positive in one case, and negative in one case. The cephalin test was 11 times positive and twice negative. In one case it was several times negative and became positive only a few weeks before the death of the patient.

Control Cases.—In 70 control cases the 3 reactions were simultaneously performed. The Takata-Ara reaction was 11 times strongly positive and 6 times weakly positive. The cephalin test was 6 times positive, once in a case of pneumonia, twice in cases of subacute bacterial endocarditis, once in a case of nephrosis, and once in a case of multiple myeloma. The results of the Weltmann test varied in different conditions. The usefulness of this test in the interpretation of various diseases has been confirmed by Teufl,[7] Levinson, Klein, and Rosenblum,[8] Levinson and Klein,[9] Dees,[10] and Kraemer.[11] Two cases of subacute bacterial endocarditis showed a lengthening of the coagulation band, a positive cephalin test, and a negative Takata-Ara reaction. These findings suggested diffuse hepatitis. There was no jaundice present, however, and there was no reason to doubt the diagnosis of subacute bacterial endocarditis on clinical and bacteriologic grounds. In one other patient suffering from cardiac failure, the results of the three tests suggested a complicating cirrhosis. The

autopsy revealed, however, only chronic congestion of the liver. In all other control cases the results of the combined tests were not misleading.

Comment.—The simultaneous use of the 3 reactions greatly enhances their value as can be recognized by critical evaluation of the tests. None of the reactions are specific. Magath[12] concludes on the basis of the literature as well as on his own extensive experience that the Takata-Ara reaction may give positive results in a wide variety of diseases. It is not a specific test but gives usually a positive result in more advanced cases of liver cirrhosis. It is frequently positive in acute hepatitis and malignant disease of the liver. The use of the cephalin flocculation test to demonstrate active disease of liver parenchyma is, without question, very valuable. We found, however, as in the Takata-Ara reaction, a number of wrong positives (8.6 per cent). A positive cephalin test might, therefore, occasionally be misleading in a patient with questionable liver disease without jaundice. The test does not always allow a differentiation between hepatogenous and obstructive jaundice. In 13 cases of hepatogenous jaundice we found the test positive only 7 times. On the other hand, the test was positive 5 times in 26 cases of obstructing jaundice. In our series the highest number of positive reactions was found in the group of liver cirrhosis. The Weltmann reaction showed the most pronounced prolongation in cases of diffuse hepatitis and also very marked prolongation in cirrhosis. It was usually normal or shortened in cases of obstructing jaundice. These findings are in full agreement with the experience of Weltmann and Sieder,[13] Teufl,[7] and Levinson and Klein.[9] The tendency to the lengthening of the coagulation band is very strongly maintained in cirrhosis especially. Complicating processes, which otherwise would lead to a marked shortening of the band, have slight, if any, influence. The coagulation band is an unspecific reaction, however, and marked lengthening is occasionally seen in other conditions.

Summary and Conclusion.—Weltmann serum coagulation band, cephalin flocculation test, and Takata-Ara reactions in the modification of Mancke and Sommer were performed. In 52 liver cases and 70 control cases it was found that by using all 3 simple tests in the same serum sample, their value for the differentiation of liver diseases could be markedly increased. Thirteen cases of diffuse hepatitis showed a frequent and pronounced prolongation of the coagulation band, a frequently positive cephalin test, and only an occasional positive Takata-Ara reaction. In 26 cases of obstructing jaundice there was found either a shortened or normal coagulation band, an occasional positive Takata-Ara reaction, and only an occasional positive cephalin test. In 14 cases of cirrhosis of the liver a prolongated coagulation band, a positive Takata-Ara reaction, and a frequently positive cephalin test were found. Only in one out of 70 control cases did the clinical picture and result of the tests lead to the erroneous diagnosis of cirrhosis of the liver.

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PREPARATION AND PROPERTIES OF A DRY POWDERED MIXTURE OF SULFANILAMIDE AND HEMOSTATIC GLOBULIN*

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ABOUT two years ago, we isolated from rabbit plasma a fraction possessing a high capacity to clot fibrinogen.[1, 2] Solutions of this fraction diluted to a protein content of 10 per cent are stable and exhibit high thrombic activity. The material was preserved with 0.3 per cent phenol and phenyl mercuric acetate 1:20,000. Prepared in this way, hemostatic globulin could be Berkefeld-filtered and stored at 5° C. for over one year, at the end of which time the product showed only a slight loss of potency.[1, 2]

Extensive studies on the physiologic action and clinical application of hemostatic globulin were performed by Finland and Taylor and their collaborators.[3, 6, 7] Additional information was presented in the paper of Bird, McSwain, Kauer, and Glenn, introduced by Reznikoff.[5] Recently Taylor and Adams extended their studies on thrombic activity of a globulin fraction prepared similarly from other species of blood (beef, swine, and human).[4] Ferguson used hemostatic globulin in the estimation of fibrinolysis.[8, 9] An editorial in the *Lancet* has reviewed the work so far performed with hemostatic globulin isolated by us from rabbit plasma.[13]

During clinical trial, it was found that these preparations could be diluted to 1 per cent protein (1:10) before being used. Such dilute solutions still contained enough potency to stop, by local application, bleeding in normal persons and in hemophiliacs.[3-5] Studies on the stability of hemostatic globulin in more dilute solutions (than 10 per cent protein) and at different pH levels are now in progress.

*From the Lederle Laboratories, Inc.
Received for publication, June 23, 1943.

Recently, we prepared dry hemostatic globulin by the process (lyophilization) that is used for the drying of human plasma. This dried material was ground in a mortar and sterilized by dry heat at 110° C. for eight hours. The treatment did not affect the solubility of hemostatic globulin. Its thrombic activity was also found to be very little affected by this method of drying and sterilization. Contrary to this, solutions of clotting globulin are much more thermolabile, temperatures high enough for coagulation of proteins rapidly destroying their clotting capacity.

The next step of our research was to prepare a mixture of dry hemostatic globulin and sulfanilamide (U.S.P., 40 to 80 mesh crystals). Hemostatic globulin was added in the amount of 5 per cent to the sulfanilamide. The materials were thoroughly mixed by grinding in a mortar, and the mixture was then sterilized by dry heat at 110° C. for eight hours in small samples, not exceeding 5 Gm. Material prepared by this method was found to be sterile.

For testing the sterility of the powdered mixture, advantage was taken of the fact that bacteriostatic action of sulfa drugs is neutralized by para-aminobenzoic acid.[10-12] The usual liquid medium (Brewer's) employed for sterility tests, and suitable for both aerobic and anaerobic growth, was modified by the addition of 10 mg. per 100 c.c. para-aminobenzoic acid. To approximately 40 c.c. of this medium in Smith fermentation tubes were added by means of a sterile glass spoon approximately 50 mg. of the powdered mixture from each container tested. At least 3 per cent of the containers in each experimental lot were thus tested. The incubation period was seven days at 37° C. To check the broth for its suitability for use in sterility testing of sulfanilamide, one tube inoculated with the powdered mixture was also inoculated with pneumococci. Prompt growth of these cocci resulted upon incubation.

TABLE I

SHOWING THAT MIXTURES OF POWDERED HEMOSTATIC GLOBULIN AND POWDERED SULFANILAMIDE CAN BE STERILIZED WITH DRY HEAT WITH APPARENTLY NO LOSS OF POWDERED PROPERTY

	133H168 ORIGINAL HEMOSTATIC GLOBULIN	*26C2* MIXTURE OF SULFANILAMIDE AND HEM. GLOB. BEFORE STERILIZATION	*26C2* MIXTURE OF SULFANILAMIDE AND HEM. GLOB. AFTER STERILIZATION
DILUTIONS	DEGREE OF CLOTTING (CLOTTING TIME—5 MINUTES)		
1:10	++++	−−	−−
1:20	++++	−−	−−
1:40	++++	++++	++++
1:80	+++	+++	+++
1:160	++	++	++
1:320	±	±	±
1:640	±	±	0
1:1280	0	0	0

After sterilization the material was tested for clotting potency. The results of these tests, given in Table I, demonstrate that the thrombic activity of hemostatic globulin in the sterile sulfanilamide mixture remained unimpaired. In order to measure the thrombic activity, 105 mg. of the dry powdered mixture were suspended in 2 c.c. of physiologic saline solution, corresponding to a 1:40 dilution of original hemostatic globulin (10 per cent protein solution). From this suspension doubling dilutions were made. To 0.1 c.c. of each dilution in Wassermann tubes was added 0.9 c.c. of oxalated normal horse

plasma (0.26 per cent) preserved with 0.45 per cent phenol. The tubes were placed in a water bath at 37° C., and the degree of clotting was recorded at the end of five minutes.

In the table the column at the left records the clotting titer of the original hemostatic globulin, No. 133H168, prepared as a solution containing approximately 10 per cent protein and preserved with 0.3 per cent phenol and phenyl mercuric acetate 1:20,000. The middle column records the clotting titer of the mixture of dried hemostatic globulin (prepared from No. 133H168) and sulfanilamide, while the column at the right records the clotting titer of the same mixture after sterilization.

Clots are designated as follows:

++++ Solid clot; adheres to sides when tube is inverted.
+++ Firm clot; does not adhere to sides when tube is inverted.
++ Partial clot; flows along sides when tube is inverted.
+ Small clot; most of the material remains liquid.
± Strands of fibrin observed when tube is shaken.

In the preceding paragraphs we have described the method of preparation of a sterile mixture of powdered sulfanilamide and hemostatic globulin. Preparation of such a mixture became possible after it was observed that dry hemostatic globulin possessed remarkable stability at high temperatures. As stated above, dry powdered hemostatic globulin withstands eight hours' heating at 110° C., and at the end of this period shows very little loss of its thrombic activity.

SUMMARY

1. Evidence is presented that dry hemostatic globulin possesses remarkable stability at high temperatures, withstanding eight hours' heating at 110° C., at the end of which period it shows very little loss of its thrombic activity.

2. The mixture of hemostatic globulin and sulfanilamide may be of value in the treatment of wounds, since it possesses both thrombic and bacteriostatic properties.

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LABORATORY METHODS

GENERAL

A SIMPLE METHOD FOR THE CONSTRUCTION OF NOMOGRAPHS*

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FREQUENTLY it is desirable to make many rapid calculations of one general type. Nomographs have been devised for this purpose. While many ingenious ways of devising them have been offered, all depending essentially on graphic representation of logarithms, the one described here is believed to embody several distinct advantages. For other methods, as well as a general discussion of nomography, the appended bibliography may be consulted.

Nomographs are not uncommonly seen in such diverse journals as "Barron's Financial Weekly," "Educational Psychology," "Anatomical Record," and the "American Journal of Hygiene." Statisticians and actuaries use them so frequently that there is now available a "Handbook of Statistical Nomographs." Since they are a valuable tool in engineering and scientific research, a simple method for their construction would appear to be of value.

Mathematical formulas are merely expressions of addition, subtraction, multiplication, and division of variables and their constants. In Fig. 1 is shown the scheme for simple addition or subtraction of two numbers, which plan represents the fundamental device of nomography. Here scales are equally divided and evenly spaced apart. Numerical values are assigned to the scales so that any point on the middle scale has twice the value of the corresponding point on either of the other scales. For example, in the addition of 4 and 12, a line called the isopleth line, drawn from 4 on one side scale to 12 on the other side scale, crosses the middle scale at 16. To substract 4 from 16, a line is drawn from 4 on the left scale through 16 on the middle scale until it intersects the right scale, this point of intersection indicating the difference between the two numbers.

Since multiplication and division can be accomplished quite conveniently by the addition or subtraction of logarithms, this same principle can be applied to these processes. When the scales are graduated logarithmically instead of equally, the distances can be added or subtracted, giving the desired multiplication or division. For this purpose the value of any point on the middle scale is made equal to the square of the corresponding point on either of the side scales, as shown in Fig. 2. Since any selected distance on the side scales is

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Received for publication, October 2, 1942.

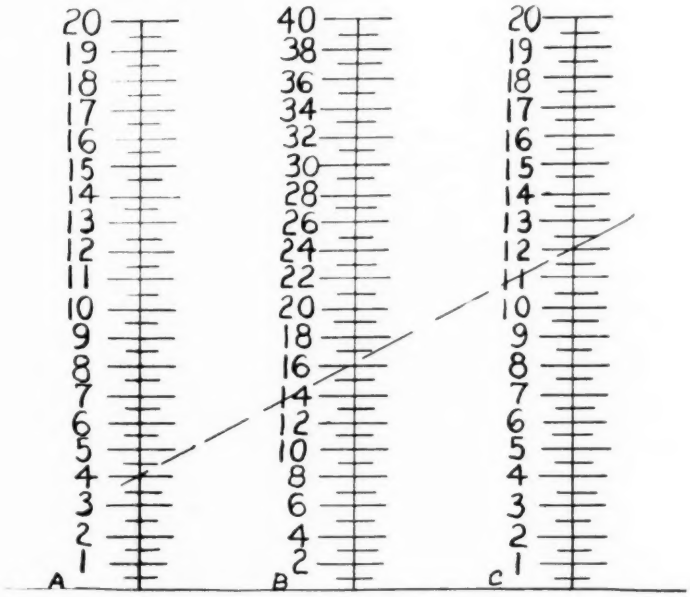

Fig. 1.—Simple nomographic addition. $A + C = B$, the diagonal line indicating $4 + 12 = 16$ or $16 - 4 = 12$.

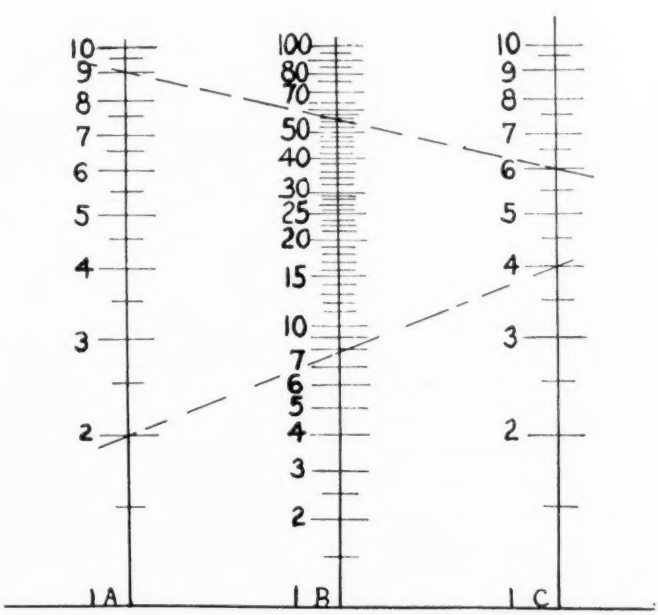

Fig. 2.—Simple multiplication, logarithmic division of scales; the product of $A \times C = B$. As shown, the diagonal lines indicate $9 \times 6 = 54$ and $8 \div 2 = 4$.

proportional to the logarithm of a number, the addition of such distances results in multiplication of the numbers. As shown, we have $9 \times 6 = 54$ and $8 \div 2 = 4$. If the side scales are not equally spaced from the middle, this displacement in itself constitutes a factor of multiplication. In the simplified system presented here intentional displacement of this kind is avoided.

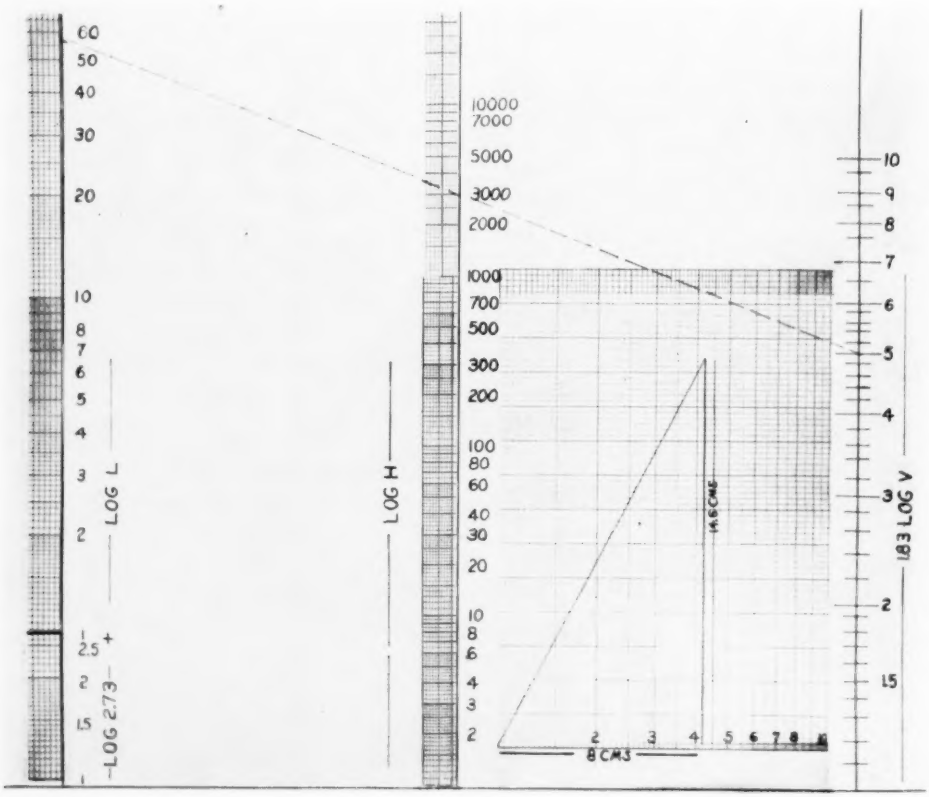

Fig. 3.—A nomograph to show $h = 2.73\ LV^{1.83}$ or $\log h = \log 2.73 + \log L + 1.83$ Log V. h = loss of head pressure in 2" pipe in ft. ÷ 1000, L = length of pipe in ft./1000 and V = velocity of liquid ft./sec.

In Fig. 3 is shown the formula and the nomograph for the calculation of the loss of pressure in a pipe in which the loss is dependent on length and velocity of the liquid passing through the pipe, $H = 2.73\ LV^{1.83}$. The novel feature here consists in the use of strips of logarithm paper held in position on stiff bristol board by cellulose tape. This obviates the laborious and tedious division of scales and also insures greater accuracy. The center scale is equally spaced between the side scales. Further, all scales begin at the same base line of value 1, so that decimals, digits, tens, etc., are easily determined. Usually logarithms are used to solve this equation; i.e., the log of h = log 2.73 + log L + 1.83 log V. The log of 2.73 is added to the log of L by cutting a strip of log paper at 2.73, and then the regular cycles of logarithms are placed above so that 1 begins at 2.73. To obtain 1.83 log V, in the insert, 8 cm. were measured off horizontally on logarithm paper, 1.83×8 cm. vertically, and a line was drawn as shown. Each unit of length on the horizontal scale has above it a segment 1.83 times

its length. These values are transferred to the right hand scale by means of dividers. The center scale is of paper twice as finely divided as the left scale. Anyone contemplating constructing nomographs will find the several varieties of log or semilog paper available of great assistance.

In Fig. 4 is a nomograph for calculating the resistance of a parachute, in which the same system has been used. The square of V is obtained by using for the right hand scale paper that is twice as coarse as that used on the left, that is, having one cycle correspond in length to two cycles. For the center scale the paper is twice as finely divided as that used for the left scale.

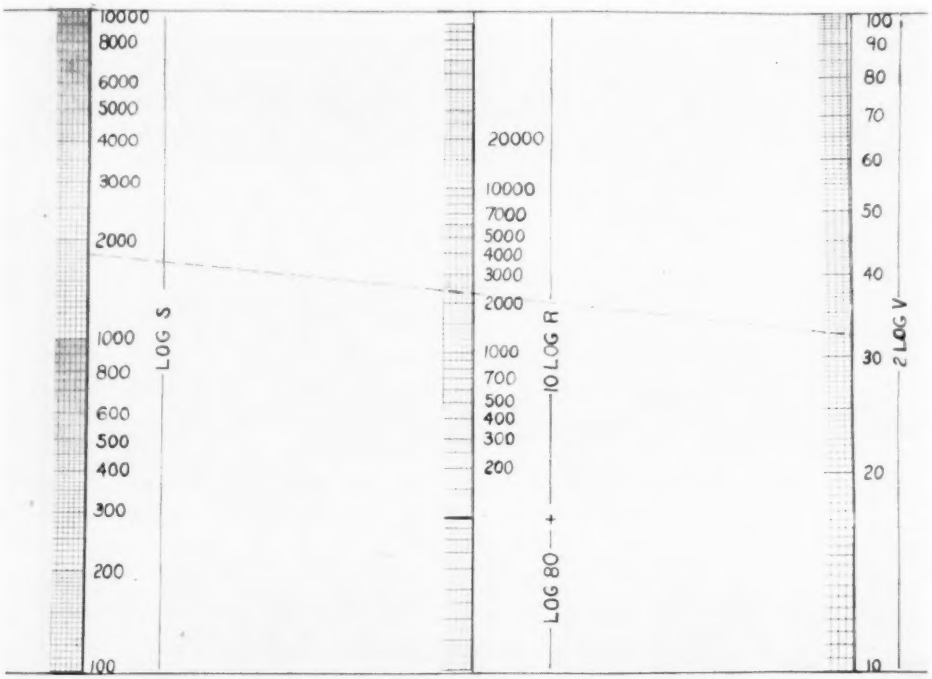

Fig. 4.—A nomograph for the resistance of a parachute as given by the formula $R = \frac{SV^2}{008}$ or $800\ R = SV^2$. R = resistance, S = area in ft.², V = velocity in ft./sec.

In Fig. 5 is shown the formula for the standardization of insulin in which there are five variables. Two variables are combined by division to obtain a reference line, this combined with a third variable to obtain a second reference line, which is left in the middle product position, and to the right is placed the C scale for division by C. Of course the constant 1.5 is taken into account by shifting A upward 1.5 logarithmic units. Finally the remaining scale gives the values of U. It should be noted that the reference lines are not divided, since their purpose is merely to give points for beginning the next operation. The value of these points as such is not needed.

The accuracy of these nomographs, just as in arithmetical multiplication, depends on the number of decimal places one keeps; that is, on logarithm paper one cycle corresponds to one decimal place. Any misplacement of the axes introduces an error. The limit of fine division varies from 25 to 100

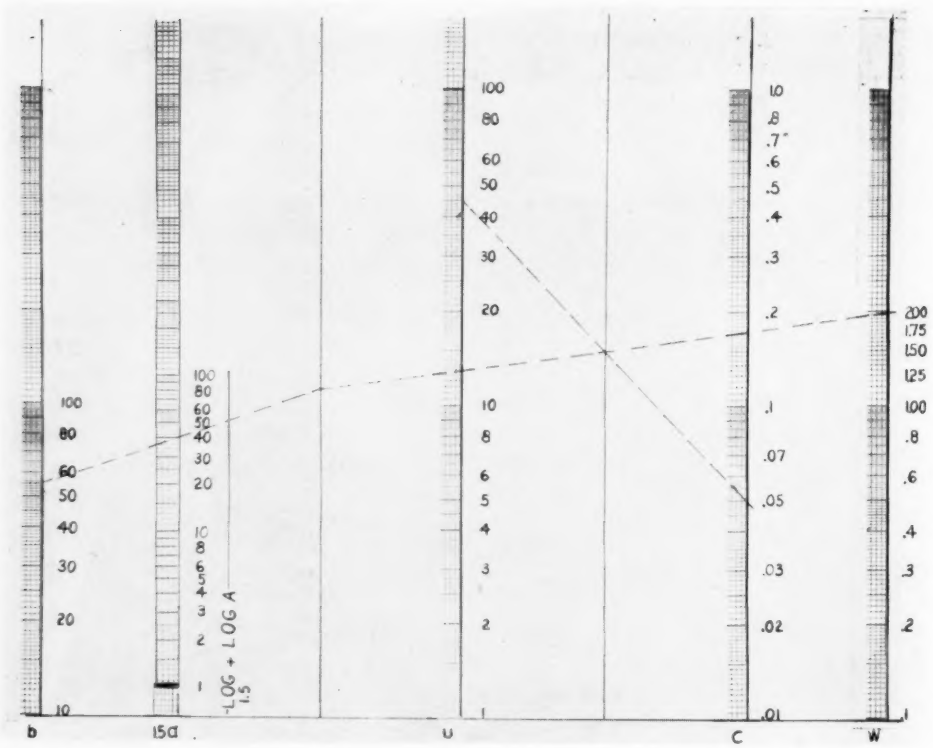

Fig. 5.—A nomograph for the standardization of insulin. $U = \frac{1.5\ AW}{bc}$, W = wt. of rabbit in kg., C = c.c. of injection, a and b, blood sugar percentages as usual in technique.

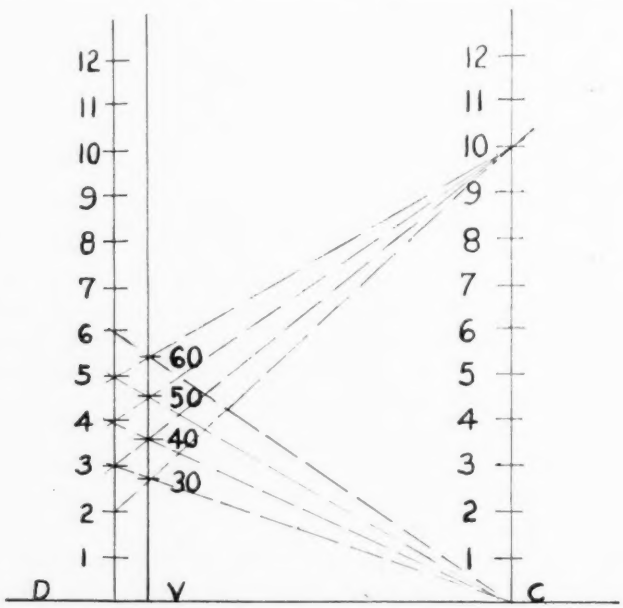

Fig. 6.—Empirical derivation of a nomogram for the simple formula V = 10 d + c, V = value in cents of a mixture of dimes and pennies, D = number of dimes, c = number of pennies.

divisions per inch, depending on the instruments used and the care exercised. Accuracy may be increased by enlarging the graph, but size ultimately reaches a conveniently usable maximum. Also, the isopleth should never slant more than 45 degrees from the horizontal, since intersections are less distinct at a greater angle. Also, it must be remembered that errors are compounded as the process is carried from scale to scale. All these may be minimized by care in construction of the nomograph, by the use of equally spaced axes when possible, having all scales begin at the same base line, and by the use of machine-ruled paper whenever possible. For permanent use and for copies, large photographs may be used under ground glass. The isopleths may then be drawn and sponged off repeatedly.

The nomographs illustrated here were selected for purposes of demonstration only, and not for specific application. While novel effects may be obtained with systems other than that employing vertical equally spaced axes, the ease and simplicity of the procedure outlined are to be recommended.

For unusual situations, such as those involving the functions of angles, recourse may be had to the empirical method as simplified in Fig. 6. Here, the result desired is the number of cents which are equivalent in value to a mixture of dimes and cents. The outer scales are evenly divided. The location of the middle scale, as well as its divisions, is determined in the following manner. Isopleths are drawn from the points zero and ten on the C scale to several points (2, 3, 4, 5) on the D scale. The intersections of the isopleths locate the position of the third scale and its divisions. The outer scales are straight vertical lines; the resulting intersections may indicate a straight line as shown in this illustration, but in other more complex relations, usually a simple curve.

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TWO NEW METHODS OF STAINING VAGINAL SMEARS*

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DURING recent years great interest has developed in studies of the vaginal secretions, based on the work of Papanicolaou. From time to time new staining techniques have been devised.

In this communication two stains† are described. The first, PGI, is recommended for studying the menstrual cycle; the second, Post Graduate II, is designed for the differentiation of malignant cells.

POST GRADUATE I FOR ROUTINE SMEARS

Fix smears immediately in equal parts of 95 per cent ethyl alcohol and ether for at least five minutes.

Run down through graded alcohols (80 per cent, 50 per cent, 30 per cent) to water. Stain in Ehrlich's hematoxylin diluted one-half with distilled water for two minutes. Wash in running water for four minutes.

Stain for two minutes in I.

Rinse quickly in water.

Stain for two minutes in II.

Rinse quickly in water.

Stain for one minute in III.

Rinse quickly in water and run up through alcohols to xylol and mount in clarite or dammar.

Staining Solutions:

1. (a) 1 Gm. ponceau de xylidine.
   99 c.c. distilled water.
   1 c.c. glacial acetic acid.
   (b) 1 Gm. acid fuchsin.
   99 c.c. distilled water.
   1 c.c. glacial acetic acid.
   Mix two parts of (a) with one of (b).
2. 2 Gm. orange G.
   1 Gm. phosphomolybdic acid.
   100 c.c. distilled water.
3. 1 Gm. fast green.
   100 c.c. distilled water.

Superficial cells stain green, deep cells a slightly darker green; highly cornified cells stain orange, and the less cornified cells, pink. Red blood corpuscles, when fresh, stain pink; when old, green.

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These studies were made possible by a grant from Mr. Jeremiah Milbank.

I wish to express my grateful appreciation to Dr. Walter Dannreuther for his constructive criticism.

†All the stains are National Aniline and Chemical Co. dyes.

Received for publication, September 29, 1942.

POST GRADUATE II FOR MALIGNANT CELLS

Fix, and run down to water, and stain in the diluted Ehrlich's hematoxylin as before.

Wash and run up through ascending alcohols (50 per cent, 80 per cent, 95 per cent).

Stain for two minutes in the following stain:

11 c.c. saturated eosin (in 95 per cent alcohol).
35 c.c. saturated orange G (in 95 per cent alcohol).
8 c.c. saturated fast green (in 95 per cent alcohol).

(In mixing this stain, add the eosin to the orange G and shake; then add the fast green slowly.)

Differentiate (agitate vigorously and rapidly) in 99 c.c. absolute alcohol with 1 c.c. glacial acetic acid in it for about one minute. Rinse in absolute alcohol to xylol and mount as before.

(This stain does not keep very long in its mixed form, and the saturated solution of orange G must be the first saturation. If the malignant cells stain too dark, add a few more c.c. of orange G.)

Superficial cells stain green, the cornified cells, red, and the malignant cells are usually red or purple.

These two stains have been used routinely in the endocrine clinic of the Department of Gynecology at the New York Post-Graduate Medical School and Hospital. They have shown a high degree of specificity and have proved eminently satisfactory in cell differentiation and diagnosis.

ON THE USE OF FIREPROOF COTTON IN BACTERIOLOGIC WORK*

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THE technique of testing bacteriostatic activity in vitro as carried out in this laboratory, and most likely also in other laboratories, involves the repeated flaming of the mouths of the test tubes and of their cotton plugs. It occurred frequently that by flaming, the plugs of neighboring test tubes, and occasionally a whole group of them, were set afire, thus not only causing an undesirable delay in the execution of the experiment, but also increasing the danger of secondary contamination. In order to avoid this disturbing and sometimes even dangerous occurrence, we suggested the use of fireproof cotton as closing wads for the tubes. This proved to be a considerable help in carrying out in vitro tests speedily and with a high degree of safety. We eventually replaced all ordinary cotton wads used for test tubes, flasks, and other containers by fireproof cotton. We have used the fireproof cotton for more than six months; since this modification was found to be most satisfactory, in our laboratory as well as in other laboratories of the research and production depart-

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Received for publication, October 13, 1942.

ments of the plant, we feel justified in recommending fireproof cotton for bacteriologic laboratory work.

Fireproof cotton shows no definite difference as compared with ordinary cotton. It stands repeated sterilization in the autoclave as well as in the hot air sterilizer. Temperatures up to 200° C. produce but a very slight browning of the fireproof plugs. Exposure to more than 200° C. or heating at 200° C. for periods longer than two hours should be avoided. The overheating produces a deep brown coloration of the plugs, which are finally disintegrated. They break easily, and a fine brown dust may cover the bottom of the glass containers.

When flamed in the flame of a Bunsen burner, fireproof plugs are only superficially charred, even if exposed for several minutes. There is no danger that the chemicals (e.g., boric acid and sodium tetraborate*) adsorbed in the process of fireproofing might exert a bactericidal or bacteriostatic activity and convey it to the medium if it inadvertently comes in contact with the plug. In order to prove this, the following experiment has been carried out repeatedly:

Sterile wads of fireproof cotton (weight 0.7 to 0.75 Gm.) were drowned in 25 c.c. papain digest broth with 0.2 per cent glucose and kept for twenty-four hours at room temperature (20° C.), in the refrigerator (+5° C.), or in the incubator (37° C.). A fourth sample was boiled in the broth for five minutes. The different samples of broth were filled in test tubes, sterilized, and inoculated with typhoid bacilli, hemolytic streptococci, *Streptococcus viridans* and pneumococci Type I. After forty-eight hours' incubation, all tubes showed as normally heavy growth as the controls which had not been in contact with fireproof cotton. The organisms were not altered morphologically and grew typically in subcultures on solid medium.

Fireproof cotton is commercially available† and less expensive than adsorbent cotton. It can, moreover, be prepared in the laboratory.‡

*U. S. Patent 2,185,695.

†We used the fireproof cotton of the Lockport Cotton Batting Company, Lockport, New York.

‡M. Leatherman, 1939, Fireproofing Christmas Trees. U. S. Dept. Agriculture. Leaflet No. 193, 1939.

A SIMPLIFIED SERUM DILUTION METHOD FOR THE QUANTITATIVE TITRATION OF PRECIPITINS IN A PURE ANTIGEN-ANTIBODY SYSTEM*

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INTRODUCTION

THE antigen dilution method of precipitin titration, based upon observation of the highest dilution of antigen yielding a visible precipitate in a constant serum-varying antigen series, continues to be used by many investigators, despite the demonstration by Cromwell,[1] in 1925, that an end point obtained by this procedure has no relationship to the antibody content of a serum. This point was emphasized again by Culbertson,[2] who suggested that the antigen dilution method is more applicable to the titration of antigen concentration than to the estimation of antibody content. This procedure is listed as an approved method of precipitin titration in most textbooks of bacteriology, however, and in the latest edition of an authoritative textbook of immunology.[3]

Our interest in the problem arose from a study of the optimal proportions method of precipitin titration, using a photoelectric method for recording the reaction rates of various antigen-antibody mixtures.[4] It was demonstrated that the galvanometer deflection produced by an antigen-antibody precipitate five minutes after mixing was directly proportional to the antigen concentration in the original mixture if there were sufficient antibodies to precipitate all the antigen. The deflection was not changed by any increase in antibody concentration. These observations were in complete agreement with those of Cromwell[1] and Culbertson[2] in that the smallest amount of antigen producing a visible precipitate is a function of the antigen concentration alone, excluding the antigen dilution procedure as a suitable titration method.

On the other hand, the more logical serum dilution procedure has not been used extensively, presumably because of the failure of the earlier workers to obtain precipitates with moderately diluted serum and because it was soon demonstrated that the serum and not the antigen provided the larger part of the precipitate. It seems justifiable to attribute some of the low titers obtained by investigators using the serum dilution method to their use of relatively high concentrations of antigen as the test dose, the inhibiting effects of excess antigen becoming more marked as the relative antigen-antibody ratios are increased in the mixtures containing the higher dilutions of serum.

It was felt, however, that a serum dilution method could be used for the accurate titration of precipitating antibodies if the inhibiting effect of excess antigen could be excluded by using, as a test dose, the smallest amount of antigen yielding a visible precipitate in the presence of excess antibodies.

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Received for publication, October 22, 1942.

EXPERIMENTAL

The Type I pneumococcic polysaccharide and the refined antipneumococcic rabbit serum (471-H-409-H) were obtained from the Lederle Laboratories through the courtesies of Dr. A. L. Joyner and Miss Frances Clapp.

Sufficient polsaccharide was dissolved in 0.85 per cent saline to make a concentration of 0.512 mg. per c.c. Progressively doubled dilutions of this stock solution were made to give a series of final antigen concentrations ranging from 0.256 mg. per c.c. to 0.0000625 mg. per c.c. after mixing with equal parts of the various serum dilutions. The progressively doubled serum dilutions, prepared in the same way, gave final concentrations ranging from 1:25 to 1:12,800.

The direct precipitin tests were performed by mixing 0.5 c.c. quantities of each reagent in Wassermann tubes. The mixtures were shaken a few times and examined for precipitate formation after standing for two hours at room temperature.

Tests for excess antigen and antibody were made on the supernatants, cleared by centrifugation after the mixtures had stood overnight in the icebox. To one-half of each supernatant was added an equal quantity of a 1:50 dilution of serum, and a solution of antigen containing 0.0005 mg. per c.c. was added to the other half.

The optimal antigen-antibody ratio, obtained by observing the antigen concentration precipitating fastest in a mixture containing a 1:100 dilution of serum, was determined by the photoelectric method.[4]

RESULTS

The results of the direct precipitation tests, presented in the upper half of Table I, are recorded as + or –, no differentiation being made as to the degree of flocculation or turbidity.

The end point in each of the "antigen dilution" titrations was the same (0.00025 mg.), although the most concentrated serum tested (1:25) contained 256 times as many antibodies as the 1:6,400 dilution. These findings were in complete agreement with those of Cromwell[1] and Culbertson,[2] and confirmed our observations obtained with the photronreflectometer.[4] The results are interpreted as eliminating the antigen dilution technique as a quantitative method for determining antibody content.

The "serum dilution" titrations, on the other hand, showed that the end points obtained with the various antigen dilutions were dependent to some extent upon the concentration of antigen used in the test. These end points would not be expected to lie in a straight line because of the well-known fact that precipitation occurs in the region of moderate antigen excess. As the concentration of the test antigen is reduced, however, the end points obtained by the serum dilution technique approach more closely to the true antibody content because of the reduction in the relative amount of excess antigen that can be precipitated by the diluted serum. Consequently it is logical to choose as the test dose of antigen the smallest concentration capable of yielding a visible precipitate with an equivalent amount or an excess of antibodies, in this instance 0.00025 mg. of polysaccharide per c.c. The highest dilution of serum precipitating this amount of antigen must therefore contain just enough antibodies to precipitate this

amount of antigen, there being no excess of precipitable antigen present in the mixture.

If the unit of antibodies be defined as the amount of antibodies equivalent to 1 μg. of antigen, as suggested by Marrack and Smith,[5] this 1:6400 dilution contained 0.25 units giving an antibody titration value of 1,600 units per c.c. of undiluted serum. The same value would have been obtained by using as test antigen the solution containing 0.0005 mg. per c.c. which reacted with the serum diluted 1:3,200. Completely misleading results would have been obtained, however, if a more concentrated test antigen had been used. For example, the antibody value of the serum, based on titration with the antigen solution containing 0.256 mg. per c.c., would have been 25,600 units per c.c., an obviously false value because this dilution of serum did not precipitate all the antigen in the mixture as shown by the presence of excess antigen in the supernatant.

TABLE I

ANTIGEN CONCENTRATION EXPRESSED IN μG. PER C.C.

DIRECT PRECIPITATION TESTS

DILUTION OF SERUM	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
1:25	+	+	+	+	+	+	+	+	+	+	+	–	–
1:50	+	+	+	+	+	+	+	+	+	+	+	–	–
1:100	+	+	+	+	(+)	+	+	+	+	+	+	–	–
1:200	–	+	+	+	+	+	+	+	+	+	+	–	–
1:400	–	–	+	+	+	+	+	+	+	+	+	–	–
1:800	–	–	–	+	+	+	+	+	+	+	+	–	–
1:1600	–	–	–	–	–	+	+	+	+	+	+	–	–
1:3200	–	–	–	–	–	–	–	–	+	+	+	–	–
1:6400	–	–	–	–	–	–	–	–	–	–	+	–	–
1:12800	–	–	–	–	–	–	–	–	–	–	–	–	–

SUPERNATANT EXAMINATIONS

1:25	A	A	–	a	a	a	a	a	a	a	a
1:50	A	A	A	–	a	a	a	a	a	a	a
1:100	A	A	A	A	(–)	a	a	a	a	a	a
1:200	A	A	A	A	A	–	a	a	a	a	a
1:400	A	A	A	A	A	A	–	a	a	a	a
1:800	A	A	A	A	A	A	A	–	–	a	a
1:1600	A	A	A	A	A	A	A	A	–	–	–
1:3200	A	A	A	A	A	A	A	A	–	–	–
1:6400	A	A	A	A	A	A	A	A	A	–	–

\+ Precipitate
– No precipitate
A Excess antigens in supernatant
a Excess antibodies in supernatant
() Optimal ratio (fastest reacting mixture)

The results of the supernatant examinations are shown in the lower half of Table I. This portion of the table can be interpreted as a series of "neutralization point" determinations, as described by Culbertson,[2] who showed that at this point "antigen and antibody combine in a constant ratio to give the maximum precipitate obtainable from the antiserum." The neutralization method gave very accurate end points except in the highly diluted antigen and antibody mixtures in which supernatant examinations would be expected to fail because the tests for residue antigens and antibodies were made on materials already doubly diluted. As expected, these neutralization points lie in a straight line and calculation from any point in this line shows that 1 c.c. of undiluted

serum contains sufficient antibodies to completely precipitate 1.6 mg. or 1600 μg. of antigen.

With serum diluted 1:50, the fastest precipitation, measured by the photron-reflectometer, occurred when an equal amount of a solution containing 0.032 mg. polysaccharide per c.c. was added. In terms of final concentration, 0.016 mg. of antigen was optimal with 0.01 c.c. of serum, indicating again that this serum contained 1600 units of antibodies per c.c.

In order to emphasize the necessity of using the least amount of precipitable antigen as test material in the serum dilution procedure, the equivalent antigen-antibody ratios, as determined by the neutralization and optimal proportions methods, are connected by a heavy line in the upper portion of Table I.

DISCUSSION

Although the data presented in this paper were obtained with a polysaccharide antigen, there is no reason for doubting its application to the titration of other antigen-antibody systems. Cromwell[1] used sheep hemoglobin and crystalline egg albumin antigens in his experiments, and Culbertson[2] did his work with crystalline egg albumin. At present we are using this method to determine the precipitin response of rabbits to the injection of beef albumin,[6] and in other experiments, we have shown that the galvanometer deflections caused by the precipitation of sheep globulin and albumin are dependent entirely upon the antigen concentration.[7]

All of the methods commonly advocated for the titration of precipitins require the use of a series of mixtures containing constant amounts of undiluted or weakly diluted serum and varying amounts of antigen. In such a constant antibody series the various end points used for estimating the precipitin content can be classified as follows: (A) the highest dilution of antigen yielding a visible precipitate, (B) the dilution of antigen producing the greatest weight or volume of precipitate, (C) the dilution of antigen producing the fastest formation of a precipitate, (D) the highest dilution of antigen completely precipitating the antibodies as indicated by the absence of antigen and antibody in the supernatant, and (E) the determination of antibody N in the precipitate at the equivalence point.

Method A has been shown to have no relationship to the antibody content and need not be considered in spite of its continued use by some observers. Method B yields results which show some relationship to the antibody content, but the method is not quantitative since the greatest weight[8] and the greatest volume[9] of the precipitate are found in the region of antigen excess. Method C is an accurate method of titration if a photoelectric method is used to record the reaction rates in the early phases of the reaction.[4] As originally described by Dean and Webb,[8] the optimal proportions procedure lacked accuracy because these authors suggested that the optimal time for reading the reaction was at the time of beginning particulation. Since the photoelectric method is a specialized procedure requiring the use of an expensive apparatus, it is not recommended for routine use in the titration of pure antigen-antibody systems. This method, however, is the only quantitative method which is applicable to the titration of precipitins in mixtures containing more than one antigen and more than

one antibody. Method D is very satisfactory for the quantitative titration of precipitins in pure antigen-antibody systems but requires centrifugation and special care in selection of the proper dose of antigen for testing the supernatants. Method E is a highly specialized procedure requiring relatively large amounts of serum.[10] The data obtained by this method, however, have been of incalculable value in the advancement of knowledge concerning the chemical factors involved in the precipitin reaction, because the antibody content of a serum can be expressed in terms of actual weight of antibody protein.

The serum dilution procedure, described in this paper, has a distinct advantage in that the titration requires only small amounts of serum; this suggests that this method would be especially valuable in the measurement of residue antibodies in absorbed sera. It must be emphasized, however, that the serum dilution technique, like the microchemical and neutralization procedures, cannot be applied to the titration of complex antigen-antibody systems.

It is unfortunate that the popularity of the antigen dilution technique of titration has resulted in its use by many investigators who often have drawn rather far-reaching conclusions from the data obtained by this method. This point can be illustrated by examination of the data presented in a paper published by Wolfe,[11] which was selected for criticism only because of its recent publication in a leading immunologic journal. This author concluded that chickens were excellent antibody producers, because high precipitin titers could be obtained frequently by a single injection of a small amount of diluted antigen. Although this conclusion may be correct, it is not justified by the data which were obtained by the antigen dilution method. His results show that the sera contained sufficient antibodies to precipitate a minute amount of antigen but give no indication as to the degree of antibody response. The use of the antigen dilution method, however, does not explain the variations in end points obtained by this author, and at least two explanations can be offered for these discrepancies. In the first place, since complex antigens (bovine, sheep, and buffalo serum) were used for immunization and testing, a number of different specific antibodies could have been formed, each antibody being present in a different concentration. A second cause for the variations may be in the use of the rather uncertain "ring test" method of setting up the reactions. The "underlayering" of 0.5 c.c. of an antigen solution with 0.1 c.c. of serum is a rather difficult procedure and introduces the possibilities of error in the measurement of the serum and in obtaining a perfect layer between the antigen and antibody solutions.

The effect of using too concentrated antigen solutions in the serum dilution titration method can be illustrated by a paper published recently by De Gara and Bullowa.[12] These authors, in a study of antibody production in serum sickness, were able to demonstrate precipitins in only one-half of their cases and concluded that serum sickness can occur without the development of observable precipitins. Although this conclusion may be correct, it must be noted that their method was incapable of detecting precipitins unless a very high precipitin titer had been developed by the patient. Since these workers used undiluted foreign sera as antigens, it is obvious that it would take a high concentration of antibodies to overcome the inhibiting effect of these concentrated antigens and to precipitate even a fraction of the precipitable antigen.

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SUMMARY AND CONCLUSIONS

A simplified serum dilution procedure is described for the quantitative titration of precipitins in a pure antigen-antibody system.

The titration values obtained by this method are in complete agreement with the values obtained by the "neutralization point" and optimal proportions methods.

The complete lack of relationship between the antibody content of a serum and the titration values obtained by the popular antigen dilution method of titration is discussed.

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AN IMPROVED METHOD OF STERNAL MARROW ASPIRATION*

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THIS communication concerns a description of an improvement in the technique of sternal marrow puncture and aspiration. The improvement consists of the localization of definite landmarks and of the use of a two-way valve either incorporated in the needle or as an adapter attached to the needles now in general use.

The routine technique employed in sternal marrow aspiration consists in puncturing the sternum below the angle of Louis, using the suprasternal notch as a midline landmark. We have found that by placing the middle finger of the free hand on the angle of Louis and the thumb and index fingers on the

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Aided by a grant from John Wyeth and Brother.
Received for publication, October 22, 1942.

lateral borders of the sternum in the second interspace, unmistakable and immovable landmarks are obtained. The puncture is then made below the middle finger and between the thumb and index fingers. In infants, where the puncture is performed in the manubrium, the middle finger is applied to the suprasternal notch and the thumb and index fingers along the lateral borders of the manubrium.

The sternal puncture needles now in use are superior to the original ones in that they are shorter and permit a firmer grasp in the performance of the procedure; this is of value particularly when the sternal plate is rather hard. In such patients with hard sternal plates, after the marrow is aspirated, it is difficult to remove the needle and syringe simultaneously without at times breaking the tip of the syringe or losing marrow material. It is to prevent such occurrences that this method, the use of a two-way valve, is described. This permits the removal of the syringe having the needle remain in situ until after filling the white cell pipette, and making smears. The needle is then removed.

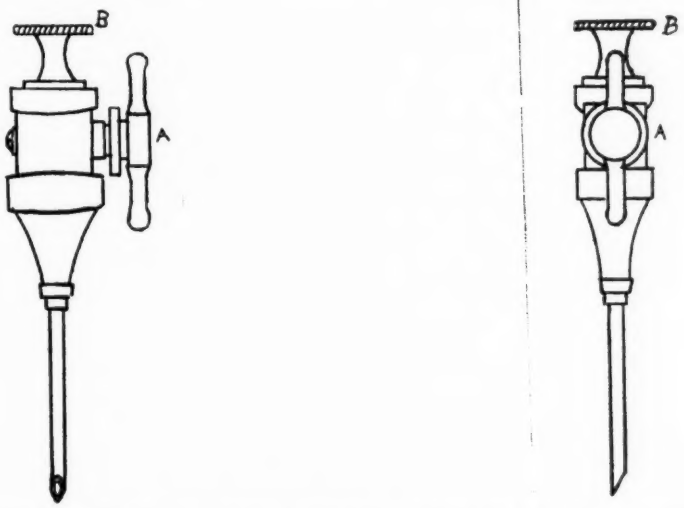

Fig. 1.—Needles used for sternal marrow aspiration. Actual size. *A*, valve control; *B*, stilet.

This may be accomplished by means of a two-way valve either as part of the needle, or as an adapter attachment. By means of the valve one may control the amount of marrow to be aspirated, since often too much marrow is sucked up, causing dilution of the marrow. Again in the so-called "dry" tap, especially in aplastic and osteosclerotic marrow after suctioning and turning off the valve, a small amount of material, sufficient to make one or two smears, may be trapped in the needle.

A three-way valve may be used for sternal transfusions, a procedure which may have to be resorted to rather often in wartime.

AN ORGAN PERFUSION PUMP*

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NEW YORK, N. Y.

AT THE present time increasing importance is being attached to studies dealing with circulatory phenomena in isolated organs. A detailed analysis of such problems necessitates the use of a simple, easily adaptable organ

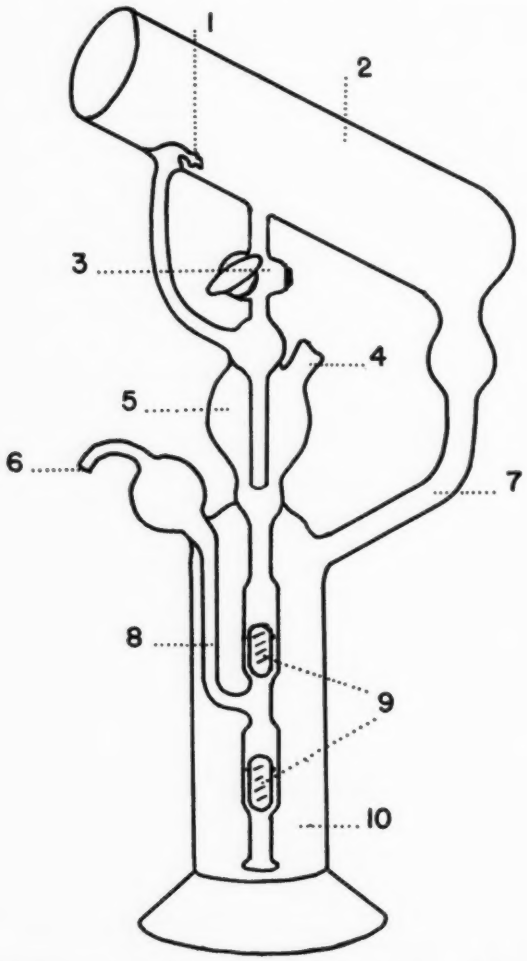

Fig. 1.—(1) cannula, (2) organ chamber, (3) by-pass, (4) outlet to pressure gauge, (5) compression chamber, (6) outlet to rubber bulb, (7) return tube, (8) pulsating fluid column, (9) gravity valves, (10) reservoir.

perfusion pump. The apparatus to be described is a single unit, made of glass and designed for sterile procedures. The mechanism is relatively inexpensive

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Received for publication, October 27, 1942.

and well adapted for use in small laboratories. All necessary adjustments of systolic, diastolic, and pulse pressure, pulse rate, etc., can be made and changed at will without interrupting the continuous flow.

Perfusion fluid flow is controlled by the action of two ground-glass, gravity valves, under the influence of a pulsating fluid column interposed between the valves. A by-pass stopcock allows any amount of the perfusion fluid and all air bubbles to be shunted and gives precise regulation of pressures. If finer adjustment is desired, the stopcock can be grooved slightly so that the fluid will flow through the grooves when the cock is turned. The necessary elastic properties can be regulated by varying the amount of air in the compression chamber. A pressure gauge is connected to the outlet of this bulb. Filters (glass, silica, platinum mesh, etc.) can be inserted at any point in the return tube or at the bottom of the reservoir.

The rate and amplitude of pulsations are regulated by the rate and degree of compression of a sturdy rubber bulb by any motor mechanism. We have, for example, adapted a sewing machine head to serve this purpose. The systolic pressure is related directly to the extent to which the rubber bulb is compressed. The diastolic pressure may be altered by changing the diameter of the opening in the by-pass. Pulse pressure, therefore, can also be changed at will.

The capacities of the different parts of the pump may be modified to suit individual needs. We have found these to be satisfactory for our work: reservoir, 300 c.c.; organ chamber, 400 c.c.; and compression chamber, 50 c.c.

The blood vessel of the organ to be studied is attached to a cannula and then by a short length of gum rubber tubing to the cannula of the organ chamber. The latter cannula can be filled previously with perfusion fluid by closing the stopcock and applying the drive mechanisms for a few strokes.

The apparatus has been used successfully for studies on perfused endocrine organs, and these will be described in another paper.

THE EFFECT OF FREEZING ON ERYTHROCYTES*

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EDWARD R. MUGRAGE, M.A., M.D., DENVER, COLO.

INTRODUCTION

THE statement most commonly made by authorities on hematology is that freezing destroys the human erythrocyte. Since it seemed to us theoretically possible to preserve red blood cells by quick freezing, we were stimulated to study the responses of the erythrocyte to this procedure.

FUNDAMENTAL CONSIDERATIONS

It is possible to freeze and thaw the red blood cell with very little cellular destruction. The speed of freezing and thawing and the concentration of certain sugars added to the blood are fundamental in the preservation of the human erythrocyte by freezing.

TECHNIQUE

Most of the results to be reported were on the human erythrocyte. Blood was drawn aseptically and mixed with dry sodium citrate so that the final concentration of the anticoagulant in the blood was 0.5 per cent. The freezing mixture consisted of ethyl alcohol and solid carbon dioxide which attains a temperature of approximately –75° C. One-half c.c. of blood and 0.5 c.c. of 20 per cent glucose were placed separately into an aluminum cup, 1 mm. in thickness, which is about the size of a water glass. The blood and sugar solution was mixed just before the cup was partially immersed in the cold bath. The best results were obtained if the freezing time was between 3.5 and 5 seconds. We found 1.0 c.c. of mixed blood and glucose the ideal amount of material to work with under the conditions of our experiment. Thawing was accomplished by dipping the cup for 1.5 to 2 seconds in a water bath held at 75° C. In order to study the blood over prolonged periods of time, 1.0 c.c. amounts were frozen, thawed, and pooled. The blood in the pooled specimens was kept refrigerated at 6° C. in sterile flasks. These were allowed to reach room temperature during a one-hour period at the same time each day. The red cells were then counted and the hemoglobin determined. The same pipet and counting chamber were always employed for each sample. Hayem's diluting fluid was used. The hemoglobin was determined by the Sahli technique on the supernatant fluid from a small specimen removed from the flask. The Sahli instrument had been standardized against the Van Slyke oxygen capacity method. In the initial hemoglobin determinations, the supernatant fluid contained so little hemoglobin that the usual Sahli technique could not be used. These initial estimates of hemoglobin were arrived at by color comparisons with samples of the original blood which was hemolyzed to known concentrations of hemoglobin by the addition of distilled water. Fifty milligrams per cent of sulfathiazole was added to both the frozen and control specimens to help

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Received for publication, October 29, 1942.

maintain sterility although every attempt was made to carry out the entire procedure aseptically. Certain studies carried out by us and several reports in the literature indicate that the addition of this small amount of sulfa drug has no deleterious effect on the blood.

RESULTS ON HUMAN BLOOD

As a result of this procedure 97 to 98 per cent initial preservation was achieved in terms of hemoglobin remaining in the red blood cells and over 99 per cent preservation in terms of cells not destroyed. This may indicate a certain amount of leakage of hemoglobin without destruction of the cell. The hemoglobin concentration in the cell was definitely less than one would expect on the basis of cell count. This is illustrated in Chart I, which represents a typical pooled sample from 20 similar samples of frozen blood followed daily by red cell counts and hemoglobin determinations. The scales on the chart are constructed so that the cell count and the hemoglobin concentration are equivalent.

CHART I

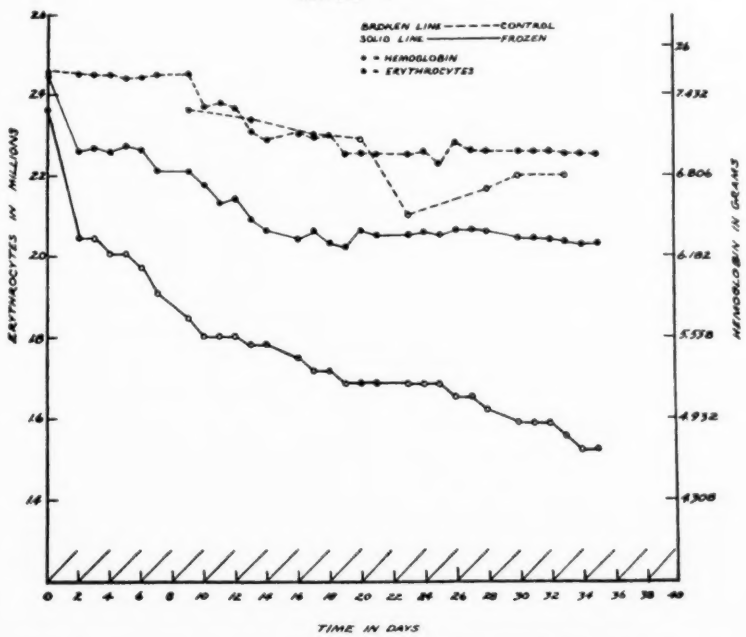


Chart I.—Pooled frozen and control specimens of human blood followed daily by red blood cell counts and hemoglobin determinations.

There was an initial fall in both the cell count and cell hemoglobin in the specimen that was frozen and thawed. After forty-eight hours this leveled off so that a moderately constant relationship was maintained in comparison with the control specimen, which differs only in not having been frozen. Microscopic study always revealed the presence of shadow cells or cells with a much reduced hemoglobin concentration. This was not found in the control.

RESULTS ON OTHER BLOOD

We have made a few studies on the blood of sheep, dogs, and rabbits. The initial results paralleled those found in human blood. The deterioration was

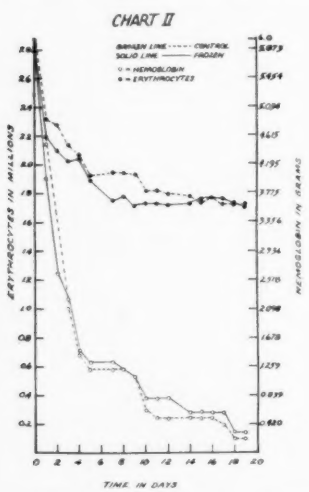
much more rapid, however, especially in respect to hemoglobin leakage, which took place at an accelerated rate even in the control specimen. The accompanying charts illustrate these points.

Unlike the experiments on human blood, however, no attempt has been made to work out the ideal technique for freezing and preservation of the different animal bloods. The conditions found best for the preservation of human red blood cells may not apply to any other blood.

PROPERTIES OF FROZEN BLOOD

Of the many properties of blood we have compared only a few. The oxygen-carrying capacity was unimpaired. Previously frozen cells can be typed with the same ease and accuracy as the unfrozen blood.

Studies on the fragility of the cells revealed peculiarities that depend on the glucose concentration of the specimen. The fragility decreases as the sugar concentration increases, even after the cells were thoroughly washed. Suffice it to say that the fragility of the frozen and unfrozen cells was identical within 0.02 per cent sodium chloride and significantly lower than the same blood not previously mixed with glucose. The whole subject of the fragility of red blood cells suspended in glucose requires further elucidation.



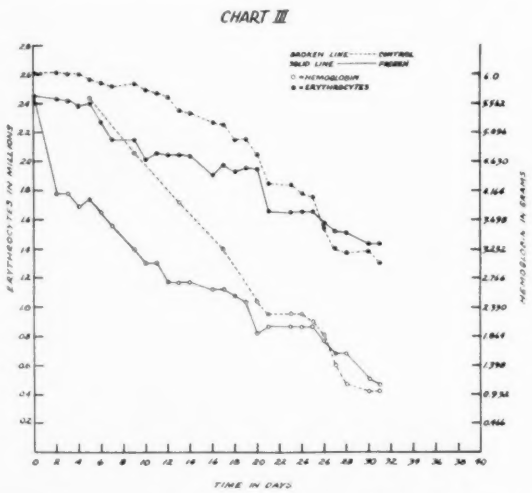


Chart II.—Pooled frozen and control specimens of dog blood followed daily by red blood cell counts and hemoglobin determinations.

Chart III.—Pooled frozen and control specimens of rabbit blood followed daily by red blood cell counts and hemoglobin determinations.

OTHER TECHNIQUES EMPLOYED

Many freezing and thawing techniques were tried but discarded, because the results were not as good as with the procedure which has been described. The blood was sprayed on solid carbon dioxide; small drops were frozen; copper and glass instead of aluminum vessels were used. Blood was also frozen in the ice compartment of an electric refrigerator at –12° C. While there was little hemolysis with this latter procedure if thawed immediately after freezing, destruction of red blood cells took place comparatively rapidly if the blood was kept frozen any length of time.

To thaw, the frozen material was added slowly to fluids of various compositions kept at temperatures ranging between 40° C. and 50° C. or allowed to melt at room temperature. This latter technique always resulted in complete hemolysis except in the case of the blood frozen in the ice compartment.

One might think that freezing of the blood more rapidly, say in one to two seconds, would give better preservation. This was tried but the results were highly unsatisfactory. A mixture of acetone and solid carbon dioxide achieves this quicker freezing. With this mixture, the blood can be frozen in 1.5 seconds. While the temperature of this mixture and our usual one of alcohol and carbon dioxide are the same, the difference in freezing time can probably be explained by the insulating effect of the carbon dioxide bubbles in the alcohol. This phenomenon of bubbling is not observed with the acetone-carbon dioxide mixture. Liquid air was also tried, but it is difficult to handle and the freezing time is about the same as with the alcohol-carbon dioxide mixture.

If we mixed 20 per cent glucose and the blood in equal amounts and allowed this mixture at least twenty-four hours to reach equilibrium before freezing, the results were never as good as when the blood and glucose were mixed within thirty seconds of freezing.

The concentration of plasma does not seem to be a vital factor in preservation. Equally good results were obtained with washed cells resuspended in glucose.

A final concentration of glucose in the blood between 9 and 11 per cent gave good results. More or less sugar resulted in a larger cell destruction on freezing. We routinely employed a 10 per cent final glucose concentration. Galactose, fructose, and sucrose gave less satisfactory results.

While several anticoagulants were quite satisfactory, we consistently used sodium citrate because it is cheap and easy to make up in the desired concentration.

STORAGE AND DRYING IN THE FROZEN STATE

Red blood cells frozen at –75° C. and stored at –12° C. for one hour in the ice compartment of an electric refrigerator invariably are completely hemolyzed on rapid thawing. We have made some attempts to determine the critical temperature at which cells are destroyed. Our experiments suggest that temperatures higher than about –40° C. result in a hemolysis of the erythrocytes. Storage of the blood in a frozen state at the temperature of solid carbon dioxide indicated that there is no deterioration during the first three days of storage. Technical difficulties have made the studies over longer periods inaccurate, but the results to date indicate a slow deterioration in the frozen state from the third to the sixteenth day.

Our few attempts to dry the cells from the frozen state on the cryochem apparatus always resulted in failure. While it is possible to preserve the cell structure when the blood is converted back to a liquid, the cells were practically completely devoid of hemoglobin. They were also extremely fragile and unless care was used in mixing the solid residue and the fluid, even the cell structure was completely destroyed.

SUMMARY

By controlling the freezing and thawing time as well as the concentration of glucose, it was possible to freeze human blood with practically no destruction of the erythrocytes. There seemed to be some leakage of hemoglobin through the cell membrane. This occurred mostly during the first two days following thawing. Pooled specimens of blood that were frozen and thawed deteriorated no more rapidly than the controls after the first forty-eight hours. The same techniques applied to several animal bloods have not been as successful as with human blood. The oxygen-carrying capacity and the ability to type the blood were not altered by freezing. The fragility of the frozen and unfrozen cells was the same. Fragility was decreased in proportion to the per cent of glucose in the blood, however.

Cells frozen at –75° C. were hemolyzed if stored in the ice chamber of an electric refrigerator. We have not found drying from the frozen state successful to date. While it was possible to preserve cell structure, the cells contained practically no hemoglobin.

A STRAIN OF BACTERIUM TYPHOSUM INHIBITED ON BISMUTH SULFITE AGAR*

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THE superiority of bismuth sulfite agar[1, 2, 3] for the isolation of *Bacterium typhosum* and *salmonella* from feces and urine is generally recognized. So much reliance is placed on this medium that the following experience is reported, not to destroy confidence in it, but as an illustration of the importance of using more than one plating medium in any attempt to isolate *Bact. typhosum* from feces.

Cultures of *Bact. typhosum* were isolated from fecal specimens from two patients and a carrier in a limited outbreak of typhoid fever in a rural community in New York state. The microorganism was present in small numbers but was found on bile salts citrate agar media,[4, 5] both before and after enrichment in tetrathionate broth. No typhoid bacilli were isolated from bismuth sulfite agar streaked with the specimens or from that with the enrichment broth. No growth was usually visible with a low power lens (×20) after forty-four hours' incubation of this medium inoculated with pure cultures. When growth occurred, the colonies were generally very small and not typical of typhoid bacilli. Other strains of this species grew characteristically on the same lots of medium.

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Received for publication, November 4, 1942.

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AN AUTOMOBILE POLLEN TRAP

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THIS is a preliminary report to introduce another simple device and new method for pollen slide exposure, namely, an automobile pollen trap. This trap has been in use since April 1, 1941; daily 24-hour exposures have been made and compared with the results of our many gravity counts. Its counterpart is found in the similar types used in airplane exposures by Stakman,[1] Scheppegrell,[2] and Meier and Lindbergh.[3] C. E. Barrett[4] in 1934 reported a few automobile studies which he had made by exposing slides on the rear seat of his car, but he has not made any contributions since that time.

A DESCRIPTION OF THE TRAP

The trap is a small, oblong, somewhat funnel-shaped box with two open ends and an inner slide shelf. It is principally made up of two parts: the outer part, a covering with the widest end or base attached to the windshield; and the inner part, the shelf upon which the slide rests.

We made a cardboard model first, then placed this design on a thin sheet of aluminum to be cut out with a pair of tin cutters. The trap should be made from lightweight material so that it is not easily detached from the car when meeting strong winds. A thin sheet of aluminum about 20 by 8 inches is ideal and sufficient to make a trap.

The length of the shelf should be such as to allow the 3 inches by 1 inch slide to rest easily upon it. The shelf is a keystone-shaped piece of aluminum with the edges on opposite sides folded one-half inch from the ends at right angles to fasten it to the trap at the proper slant. The entire device is fastened together with lightweight rivets, the ends of which can be turned in with a screwdriver. Wind vents are placed at the base to release the air as it rushes through the trap. Four rubber suction cups are used to attach the trap to the windshield.

Since we have been using a convertible car, the windshield is preferred, but with slight modifications the trap could be placed on the roof or elsewhere just as easily. One precaution should be taken on the windshield attachment. Certain state vehicle codes prohibit any obstructive objects on the windshield,

Received for publication, November 9, 1942.

and in these states a permit must be obtained for application thereon. The importance of where the trap is attached was brought out by Stakman in his airplane studies, when he showed that fewer spores were caught in the George trap when attached to the strut above the surface of the lower wing than when held out of the cockpit by hand.

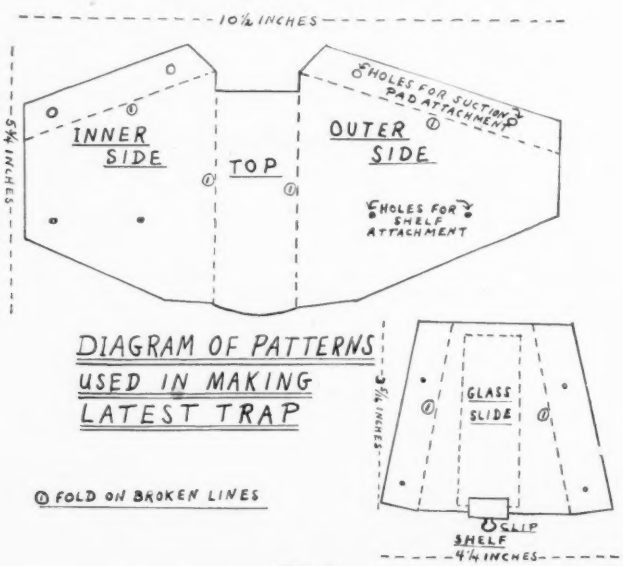

Fig. 1.

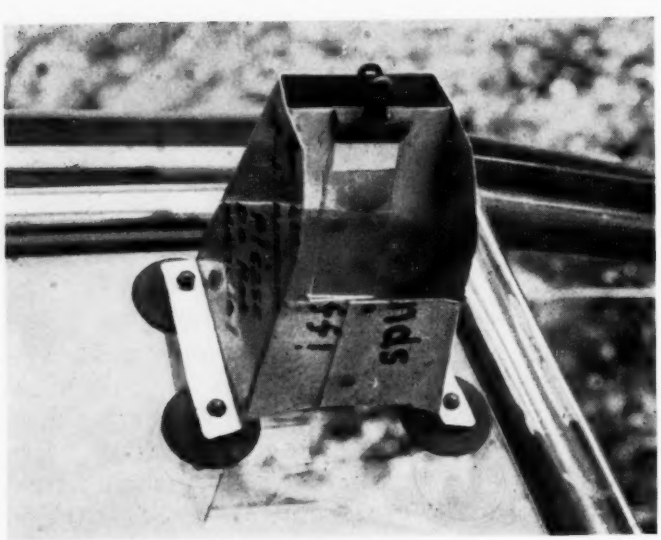

Fig. 2.

The rubber suction cups with a small bolt and nut fastened in the center and a steel clip attached can be purchased at a five-and-ten store. The clips are removed, but can be used to great advantage in anchoring the slide to the shelf. The suction cups allow for easy removal, and the clip makes changing of the slides simple. A one-half inch strip is also turned at right angles at the base on

opposite sides. Two holes are driven through each strip and the suction cups easily bolted on. Wetting the suction surface will give a strong anchorage so that under ordinary driving ranges and meteorologic conditions, the trap will stay in place for about two weeks, after which it should be taken off and reapplied.

Because the windshields vary on different cars, each trap should be fitted to the automobile for which it is to be attached. All the angles are very important, especially the one at which the slide is exposed to the air, and also the one for the protection of the slide from the elements while the car is standing. Of course the design used now favors collection while the car is in motion.

The previous description is a design of a trap which we have used successfully and which we believe with minor changes can be universally adapted to all makes of cars; however, we are constantly experimenting, changing the design and angles to further our knowledge, and hoping for more improvement. We have made many counts with the slide shelf at various angles as it meets the onrushing air, from 30 to 170 degrees. Fig. 3 shows the angle about 75 degrees, but our latest model is placed at 100 degrees. It is our impression that a good deposit of grains is obtained when the slide shelf is placed in a position to meet a great deal of wind resistance.

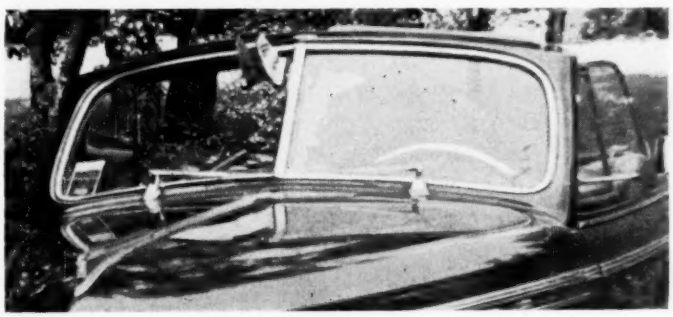

Fig. 3.

Our device has been easy and cheap to construct, a fact we wish to emphasize. It is felt that this trap has the following advantages and with more use can be further developed:

(1) *Simplicity.*—Thus slides for study are easily obtained.

(2) *Mobility.*—The trap can cover any area desired.

(a) This makes pollen studies possible in isolated districts large or small, places in which to look for new types of grains.

(3) *Rapid Sampling.*—This factor will vary with speeds and the exposure angle in the trap. It is from 30 to 150 times faster than the gravity collection.

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CHEMICAL

SPECTROPHOTOMETRIC DETERMINATION OF EVANS' BLUE IN SERUM*

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THE absorption spectra of Evans' blue and oxyhemoglobin are related in such a manner that the dye is readily determined with a spectrophotometer[1] or photoelectric colorimeter[2] in sera showing varying degrees of hemolysis. Constant turbidity introduces no error, but variable turbidity is recognized as a source of uncontrollable errors.[1] In human sera hemolysis is rare if the blood samples are protected with oil, and turbidity is not a common problem. But with dog sera these difficulties are encountered separately or together, particularly in experiments which extend over a period of six hours or more and involve departures from normal conditions.

Results of a preceding study[3] made it possible to choose conditions under which errors due to hemolysis and turbidity can be corrected simultaneously. Correction for turbidity also permits use of 0.8 per cent sodium chloride solution in place of dye-free serum as a blank in spectrophotometric readings. This is advantageous when the dye is given eighteen hours before the experiment, as in "indirect" measurement of changes in plasma volume, and when the dye-free serum is turbid while the dyed sera are clear. In the following procedure one other change in the customary technique is introduced. The sera are diluted with an equal volume of saline solution before reading. This simplifies the calibration and reduces the amount of serum required.

APPARATUS

In the present study a Beckman spectrophotometer[4] equipped with four precision cells of 1 cm. depth was used. Readings in the infrared were required to establish a basis for the simplified technique given below, which is within the range of other instruments.

The cells of the Beckman instrument require 3 c.c. of solution. For admitting to them equal volumes of serum and 0.8 per cent sodium chloride solution the author made a 1.5 c.c. Ostwald-type pipette from capillary tubing having an outside diameter of 7 mm. and a bore of 1.7 mm. The delivery tip of the pipette is 75 mm. long, tapers from 5 mm. to 1 mm. in outer diameter, and has a fine bore. Calibration with water shows that delivery is reproducible to 0.001 c.c.

TECHNIQUE

As in previous methods, paraffin oil is used in the syringes and 15 × 85 mm. specimen tubes to minimize hemolysis. The clotted specimens are centrifuged thoroughly. Attaching a rubber tube to the pipette for suction enables

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Received for publication, July 15, 1943.

one to hold the specimens at the level of the eyes, so that serum from small samples can be measured without waste or danger of including cells. While the tip of the pipette passes through the oil, a few bubbles of air should be blown through it. When the pipette has been filled, oil and serum are carefully wiped from the outside and the volume is adjusted.

To 1 volume of each dyed serum one adds 1 volume of 0.8 per cent sodium chloride solution. The extinction (optical density) of the diluted sera is then measured at 750 and 630 mμ. Either dye-free serum diluted in the same manner, or saline solution, may be used as a blank.

CALIBRATIONS

Place 1 volume of the same dye-free serum into each of two cells. To the first cell add 1 volume of saline solution and mix. To the second cell add 1 volume of solution containing 12 mg. of Evans' blue and 8 Gm. of sodium chloride per liter. Using the first cell as a blank, measure the extinction at 750 and 630 mμ. To obtain the coefficients used in calculating Evans' blue concentrations in mg. per liter of undiluted serum, divide the extinction readings by 12. The dilution is ignored, for dyed sera are diluted with saline to the same extent that the saline dye solution is diluted with serum.

As a stock solution of the dye, the one recommended by Gibson and Evans is used. With freshly distilled water and 0.3 Gm. of the dye one prepares 100 c.c. of solution. This is filtered through a sintered glass filter tube, sealed up in ampoules, and autoclaved. The same solution is used for intravenous injection and for preparing the calibrating solution.

The oxyhemoglobin calibration is carried out in the same way, by substituting a saline solution of oxyhemoglobin for the dye solution. The exact concentration of oxyhemoglobin is immaterial, for only the ratio between optical densities at 750 and 630 mμ is needed. The oxyhemoglobin solution mentioned in Table I was prepared by diluting 2 c.c. of citrated blood of a normal dog to 100 c.c. with distilled water, adding 0.8 Gm. of sodium chloride after laking, then filtering through Whatman No. 42 paper.

The turbidity correction is best determined with sera of the type to be encountered in the contemplated study. After diluting the sera with equal volumes of saline, which is also used as the blank, one measures the extinction at 750 and 630 mμ and calculates the ratio between the two values. Somewhat more detailed data required in the present study are shown in Table II. The extinction of eight turbid unhemolyzed dog sera was determined as stated at five or more points between 1000 and 600 mμ. The logarithms of the extinctions were plotted against the negative logarithms of the corresponding wave lengths, and the resulting straight lines[3] were extrapolated to 577 mμ, where traces of oxyhemoglobin interfere with direct measurement of turbidity. From these lines the extinction values shown in Table II were taken. The sera are arranged in decreasing order of turbidity as measured at 630 mμ.

PRINCIPLES OF CALCULATION

The following simultaneous equations present the general problem of determining the concentrations x, y, and z of three substances by spectrophotometry.

$$a_1x + b_1y + c_1z = E_1 \quad (1)$$
$$a_2x + b_2y + c_2z = E_2 \quad (2)$$
$$a_3x + b_3y + c_3z = E_3 \quad (3)$$

One measures the extinction, E_1, E_2, and E_3 of a solution of the three substances at three suitably selected points in the spectrum. The coefficients a, b, and c are determined by calibrating with each substance separately at each of the three points selected. In the present instance E_1 is measured at 750 mμ, E_2 at 630 mμ, and E_3 at 577 mμ. Let y equal the concentration of Evans' blue in mg. per liter of undiluted serum, while x and z represent the oxyhemoglobin concentration and turbidity in any desired units. By calibration one finds that the absorption of the dye at 750 mμ is negligible if the instrument excludes stray light and a concentration of 12 mg. of dye per liter is not greatly exceeded. Thus b_1 is for our purposes zero. Furthermore, one of the reasons for choosing the wave length of 750 mμ is that this makes the ratios a_2/a_1 and c_2/c_1 nearly the same. If these ratios were equal and were represented by k, equations 1 and 2 would yield the following solution for y when b_1 is zero.

$$y = \frac{E_2 - kE_1}{b_2} \quad (4)$$

TABLE I

EXTINCTION OF OXYHEMOGLOBIN IN DOG SERUM

CELL NO.	SERUM C.C.	OXYHEMOGLOBIN SOLUTION C.C.	SODIUM CHLORIDE 0.8% C.C.	EXTINCTION, 1 CM. CELLS				RATIOS OF EXTINCTIONS		
				750	630	577	540	750	630	577
1	1.5	0.0	1.5	blank						
2	1.5	0.5	1.0	.008	.013	.492	.471	.163	.264	10.0
3	1.5	1.0	0.5	.011	.020	.983	.943	.112	.203	10.0
4	1.5	1.5	0.0	.017	.029	1.50	1.43	.113	.193	10.0

TABLE II

EFFECT OF TURBIDITY AT DIFFERENT WAVE LENGTHS

SERUM NO.	EXTINCTION, 1 CM. CELLS			RATIO OF EXTINCTIONS		
	750	630	577	750	630	577
4	.350	.530	.660	1	1.51	1.89
5	.315	.500	.630	1	1.59	2.00
1	.330	.490	.610	1	1.48	1.85
6	.225	.348	.435	1	1.55	1.93
2	.215	.335	.420	1	1.56	1.95
3	.145	.227	.290	1	1.57	2.00
7	.138	.225	.285	1	1.63	2.07
8	.113	.181	.245	1	1.60	2.17
Average ratios				1	1.56	1.98

In the present case the relationship between the coefficients in equation 3 is such that this simplified solution is sufficiently correct. To demonstrate this, we substitute actual calibration values for the coefficients of x, y, and z in equations 1, 2, and 3. For a_1, a_2, and a_3 we substitute the last set of ratios in Table I. For c_1, c_2, and c_3 we substitute the average ratios at the foot of

Table II. For b_1, b_2, and b_3 we enter values found by calibrating with Evans' blue in the manner described, at 750, 630, and 577 mμ.

$$.113\ x + .0000\ y + 1.00\ z = E_1 \qquad (5)$$

$$.193\ x + .0389\ y + 1.56\ z = E_2 \qquad (6)$$

$$10.0\ x + .0280\ y + 1.98\ z = E_3 \qquad (7)$$

Equation 5

$$z = E_1 - .113\ x \qquad (8)$$

Substitute (8) in (6) and (7)

$$.017\ x + .0389\ y = E_2 - 1.56\ E_1 \qquad (9)$$

$$9.776\ x + .0280\ y = E_3 - 1.98\ E_1 \qquad (10)$$

It is then necessary to divide equation 10 by 575 to obtain the same coefficient for x as in equation 9, and as a result the coefficients of other terms in equation 10 become almost negligible. The final solution is:

$$.03885\ y = E_2 - 1.5634\ E_1 - .00174\ E_3 \qquad (11)$$

$$y = \frac{E_2 - 1.5634\ E_1}{.03885} - .045\ E_3 \qquad (12)$$

Equation 12 differs from equation 4 principally in the presence of an additional term. But if E_3, the extinction at 577, were 2.0, corresponding to 1 per cent transmittance, y, which represents mg. per liter of Evans' blue in serum, would only be changed by 0.09, a matter of less than 1 per cent if the value of y is 10 or over. The value of c_2/c_1 in equations 5 and 6 is 1.56 and that of b_2 is 0.0389. In equation 12 these values have only been altered a few parts per thousand. Whether one should use equation 4, or carry out all calibrations at three points and derive equation 12, is purely a question of whether the two procedures yield as nearly identical results when other types of apparatus and different experimental conditions are employed.

SOURCES OF ERROR

The least complicated determinations, namely, the calibrations with Evans' blue, may be considered first. The same amount of the same serum is present in the blank and the sample under observation; hence, differences in hemolysis and turbidity should not exist. Extinction readings at 750 mμ or longer wave lengths show that this cannot always be taken for granted, for differences in the manner of pipetting or mixing may produce differences in turbidity. If the reading at 750 mμ is zero, the term kE_1 drops out of equation 4. The exactness with which the extinction at 630 mμ, E_2, can be measured, and the constancy of the calibration factor b_2, remain as the limiting factors in accuracy. It is known that extinction measurements between 0.300 and 0.700 are very reproducible with a number of instruments. In the present method the readings will be near the midpoint of this range if the undiluted serum or the calibrating solution contain 12 mg. of dye per liter. This concentration is therefore ideal, and concentrations of 6 to 18 mg. per liter are satisfactory.

The constancy of b_2 was studied in some detail. Four sera were obtained in the course of a week from each of four normal dogs receiving the same diet. Three concentrations of the dye were determined in each of the 16 sera. The data in Table III thus correspond to 48 recoveries of added dye, reported in terms of the observed extinction or optical density. Dividing the values in each line by the concentration of dye indicated in the first column would yield

TABLE III

EXTINCTION OF EVANS' BLUE IN VARIOUS SERA AT DIFFERENT CONCENTRATIONS

MG./L OF DYE IN SOLUTION ADDED TO SERUM	EXTINCTION AT 630 mμ, IN 1 CM. CELLS, AFTER ADDITION TO AN EQUAL VOLUME OF SERUM								MEAN	S.D.
	DOG 1	DOG 2	DOG 3	DOG 4	DOG 1	DOG 2	DOG 3	DOG 4		
3	.121	.106	.117	.117	.114	.107	.108	.115	.113	.005
6	.239	.223	.237	.238	.233	.231	.233	.228	.233	.005
12	.468	.459	.463	.478	.469	.471	.470	.462	.4675	.006
3	.119	.118	.119	.118	.115	.119	.119	.118	.118	.0004
6	.237	.237	.238	.234	.230	.238	.238	.232	.2355	.003
12	.468	.471	.474	.464	.456	.476	.467	.452	.466	.008
Mean of four determinations with 12 mg./L in each animal					.465	.469	.469	.465		
Mean of 16 determinations with 12 mg./L in 4 animals									.467	.007

b_2. With the largest concentration of dye employed the mean result was nearly the same for each animal as for the entire series, and the standard deviation of the series is only 1.5 per cent of the mean. In these 16 determinations the technique was precisely as described above for calibration with the dye.

With smaller concentrations two procedures were used. The data in the first two lines of Table III were obtained by using the technique as described, with the single change that the calibrating solutions contained 3 and 6 mg. of dye per liter instead of 12 mg., the sodium chloride content being constant. In the second set of determinations, only the solution containing 12 mg. of dye per liter was added to serum. The extinction having been read, 1.5 c.c. of the sample was transferred to the next cell and mixed with 1.5 c.c. of a diluent consisting of equal parts of the same serum and saline. The extinction was read, and dilution by the same method was repeated once more. Repeated diluting and transferring caused a very slight increase in turbidity, detected at 750 mμ, for which corrections of .003 or less were subtracted. In contrast with the first set, results with the lower concentrations of dye were quite constant, as indicated by the standard deviation. In either set the extinction was very nearly halved when the highest concentration was halved. In a study in which one uses a number of animals under similar conditions it is therefore satisfactory as well as convenient to use an average of four or more calibrations with representative sera of the group and with one reproducible calibrating solution. When pure solutions of the dye were examined, the extinction at the same concentration and wave length was about 6 per cent greater than in serum, and the specific extinction was perfectly constant for different concentrations. If an instrument does not give the latter result with pure solutions, calibrations with various concentrations of the dye of course become necessary.

The accuracy of the correction for hemolysis and turbidity remains to be considered. This is a question of the constancy of k in equation 4. Considering hemolysis first, we find the necessary data in Table I. Whether the extinction values at 750 mμ are multiplied by 1.5 or 2.0, the error in predicting the values at 630 mμ does not exceed .005. E_1 is so small that variations in k are unimportant, provided that one uses concentrations of the dye that give a relatively large value for E_2.

When one dilutes the filtered oxyhemoglobin solution prepared as directed for calibration, the ratio of extinctions at 574 and 620 mμ, or 540 and 620 mμ, which is reported as 40:1 in the literature,[1, 2] progressively falls. This may be due in part to the difficulty of avoiding turbidity completely. In any event it introduces no error in the present method. An oxyhemoglobin solution which was obviously turbid gave a value of only 15.2 : 1 for the above ratio, but the ratio between extinctions at 750 and 630 mμ was near the average of those in Table I.

A serious and incompletely understood source of error related to hemolysis in sera from actual experiments is that a hemolyzed dyed serum not infrequently gives a high value, quite out of line with those for preceding and following unhemolyzed dyed sera. This is true whether the present or previous methods of correction for hemolysis are used. The maximum absorption of Evans' blue is near that of methemoglobin and sulfhemoglobin, and conversion of even a few per cent of the oxyhemoglobin in hemolyzed serum to these derivatives would introduce a large error. The difficulty is probably not due to technical errors and may not be due to the above derivatives, but possible contact of the specimens with traces of oxidizing agents or hydrogen sulfide must be excluded.

TABLE IV

VALUES OF k FOUND BY MEASURING THE EXTINCTION AT 750 mμ (E_1) AND 630 mμ (E_2) IN SERA VARYING FROM EXTREME TO MINIMAL TURBIDITY

E_1	E_2	k	kE_1	E_2-kE_1	E_1	E_2	k	kE_1	E_2-kE_1
.347	.530	1.53	.531	−.001	.030	.058	2.00	.060	−.002
.340	.529		.520	+.009	.029	.060		.058	+.002
.322	.484		.493	−.009	.029	.051		.058	−.007
.254	.404	1.60	.406	−.002	.027	.046		.054	−.008
.251	.398		.402	−.004	.026	.049		.052	−.003
.244	.370		.390	−.020	.024	.043		.048	−.005
.220	.348		.352	−.004	.020	.041		.040	+.001
.201	.325		.322	+.003	.019	.039		.038	+.001
.144	.233		.230	+.003	.019	.039		.038	+.001
.135	.218		.216	+.002	.016	.034		.032	+.002
.100	.171	1.75	.175	−.004	.015	.030		.030	.000
.075	.137		.131	+.006	.015	.030		.030	.000
.058	.105		.102	+.003	.014	.028		.028	.000
.058	.100		.102	−.002	.011	.025		.022	+.003
.045	.080		.079	+.001	.011	.026		.022	+.004
.035	.060		.061	−.001	.010	.020		.020	.000
					.008	.020		.016	+.004
Mean error				.0047					.0025

With reference to turbidity, two situations may be considered. If the control serum and the dyed sera are clear or nearly so, the diluted control serum may be used as a blank. The extinction at 750 mμ will then often be negligible, but will serve as an objective check. Most of the sera in the right half of Table IV could have been subjectively classified as excellent, yet they differ considerably.

If marked turbidity is present in several sera, or in the control serum, the author prefers to make all readings with saline solution as the blank. The value of k in equation 4 was therefore studied in detail, and the results are given in Table IV. Thirty-three sera from normal dogs were diluted with equal volumes of saline and the extinctions were measured at 750 and 630 mμ with

saline solution as the blank. Postprandial as well as postabsorptive sera were included. All sera were of course dye-free. The 16 sera in the left half of Table IV and some of the clear ones in the right half came from specimens drawn and delivered under oil and contained very little oxyhemoglobin. The sera are arranged in descending order of turbidity and are divided into classes with upper limits of 0.030, 0.100, and 0.300 for the extinction at 750 mμ, or E_1. The value of k which most nearly predicts E_2 within these classes is shown, and the mean of the errors in prediction, regardless of sign, is also given. By inspection of the table, it will be noted that an error of .009, or approximately twice the mean error for the turbid sera, is only exceeded in 1 out of 33 sera. This error of .009 corresponds to 0.23 mg. of Evans' blue per liter of serum in the present method, or about 2 per cent if the ideal concentration of 12 mg. per liter is employed.

Evans' blue is removed quantitatively from pure solutions by precipitating calcium oxalate therein. The presence of protein largely prevents this, but the use of serum, as recommended in the original studies on this subject, should be continued. Optical cells used repeatedly with dyed sera presently show extinction values as large as 0.020 at 620 mμ, when filled with saline after thorough rinsing, and should therefore be cleaned with chromic acid or a detergent between determinations. The extinction of the dye at 615 to 630 mμ is not notably altered by changes in pH from 3.0 to 8.0, but stronger alkali changes the color. Sodium hydrosulfite destroys the color in alkaline and weakly acid solutions, an intermediate red stage being noted in the latter.

SUMMARY

Technique for spectrophotometric determination of Evans' blue (T-1824) in serum, with a single correction for both turbidity and hemolysis, is described, and sources of error are examined. When the concentration of dye is 12 mg. per liter and no correction for turbidity or hemolysis is needed, the probable error of individual determinations is 1 per cent. When the correction for hemolysis and turbidity is large, a total error of 4 per cent is possible. To exclude it an adequate number of observations must be made under uniform experimental conditions. Badly hemolyzed dyed sera not infrequently yield unreliable results suggesting the presence of methemoglobin. Any technical errors that could result in formation of this compound or sulfhemoglobin must be avoided. Advantages of the dye over those previously used are well-known; its principal disadvantage is the readiness with which it is adsorbed on glass, calcium oxalate, and protein.

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DENSITY FINDINGS WITH HEPARIN IN BLOOD, PLASMA, AND SERUM*

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WHILE studying viscosities of heparinized blood we[1] found a decrease in the density with heparin. This led us to investigate further the influence of heparin added in various units to blood, plasma, and serum.

The densities were determined by pyknometry, and calculated by the equation, Density $= \frac{\text{Weight}}{\text{Volume}}$. Two Nicol tube pyknometers with capacities of 1.2517 c.c. and 2.2869 c.c. were used. The blood was obtained in clean dry 50 c.c. syringes. Adequate accuracy to measure 0.3 c.c. quantities of liquid in question was provided by the use of precision Pyrex pipettes, Mohr Measuring Type, with capacities of 0.1 and 0.2 c.c. It was found essential that the glassware employed be rigorously cleaned.

Blood was taken from three human beings and diluted 9:1 with anticoagulant solutions. Not less than 9 c.c. of blood were used for each dilution. Two, 5, 25, and 125 units of heparin (110 Murray-Best units per milligram of the sodium salt) were contained per c.c. of these blood systems. For controls, 0.2 per cent sodium oxalate, 0.2 per cent potassium oxalate, and 0.3 per cent sodium citrate in equal volumes of blood dilution were used. One portion of each of the blood systems was centrifuged at 3,000 revolutions per minute for twenty minutes, and the supernatant plasma was tested for density. All tests were run continuously within six hours following blood withdrawal. With increasing amounts of heparin, the density decreases in both blood and plasma, but not in linear proportion. Samples containing heparin show lower density than the controls. Results are compiled in Table I. Similar results, as with sodium oxalate, were obtained with potassium oxalate and sodium citrate.

Serum from two human beings, three dogs, and one sheep was obtained six hours following blood withdrawal by centrifugation at 3,000 revolutions per minute for twenty minutes. Three-tenths c.c. solutions of 10, 50, 250, and 1,250 units of heparin in distilled water and, as control, 0.3 c.c. distilled water were added to 3 c.c. serum, and pyknometry was done within three hours. In all instances the density was lowered with heparin. The decreases were more marked with higher heparin concentration, although they were never directly proportional (Table II).

Since Chargaff and Olson[2] found that protamine deionizes and flocculates heparin, we have determined the influence of various amounts of heparin on the density of a 1.5 per cent protamine sulfate‡ solution in physiologic saline. Following centrifugation of the protamine solution at 1,500 revolutions per

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‡A sample of protamine sulfate was kindly supplied by E. R. Squibb & Sons, New Brunswick, N. J.

Received for publication, April 12, 1943.

minute for ten minutes, heparin solutions were added to the decanted fluid. In each case the density was decreased by heparin.

TABLE I

IN VITRO EFFECT OF VARIOUS AMOUNTS OF HEPARIN UPON DENSITY OF HUMAN BLOOD AND PLASMA AT 27° C.

CASE	FLUID	PER C.C. OF BLOOD-ANTICOAGULANT MIXTURE 9:1				
		UNITS OF HEPARIN				SODIUM OXALATE
		2	5	25	125	0.2 PER CENT
A	Blood	1.0589	1.0588	1.0586	1.0584	1.0593
	Plasma	1.0290	1.0288	1.0284	1.0279	1.0291
B	Blood	1.0549	1.0546	1.0547	1.0540	1.0558
	Plasma	1.0241	1.0240	1.0238	1.0234	1.0242
C	Blood	1.0488	1.0489	1.0468		1.0495
	Plasma	1.0227	1.0224	1.0219		1.0233

TABLE II

INFLUENCE OF VARIOUS AMOUNTS OF HEPARIN UPON DENSITY OF SERUM OF DIFFERENT SPECIES

SERUM	TEMPERATURE °C.	UNITS OF HEPARIN IN 0.3 C.C. DISTILLED WATER ADDED TO 3 C.C. SERUM				
		0	10	50	250	1250
Human I	26	1.0232	1.0230	1.0229	1.0228	1.0224
Human II	27	1.0247	1.0238	1.0237	1.0235	1.0231
Dog I	27	1.0274	1.0269	1.0269	1.0261	1.0255
Dog II	27	1.0263	1.0261	1.0260	1.0259	1.0257
Dog III	29	1.0191	1.0189	1.0187	1.0185	1.0183
Sheep	29	1.0233		1.0224	1.0223	

SUMMARY

Heparin has a specific lowering action upon the density of the four systems of blood, plasma, serum, and protamine sulfate solution. Increasing amounts of heparin decrease the density more markedly, but not in linear proportion. Data are not, as yet, sufficiently extensive to draw any conclusions as to the mechanism of this action.

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VI. A SIMPLE AND ACCURATE METHOD FOR QUANTITATIVE DETERMINATION OF BILE ACIDS AND SALTS IN BILE

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IN 1940, in this journal, the author, in collaboration with W. A. Swalm, gave a brief, preliminary description of a simple, stalagmometric method for the quantitative determination of bile salts in urine and bile.[1] During the intervening years, this procedure has been utilized in the author's laboratory as one of the routine laboratory aids for the estimation of hepatic function in diseases involving the liver or biliary tract.[2, 3] This experience in application of the procedure in the analysis of samples of bile obtained by intubation of the duodenum has indicated some possible sources of error and the need of amplifying the original description of the technique.

APPARATUS

Fig. 1 illustrates the stalagmometer, which may be obtained from the Precision Thermometer and Instrument Co., of Philadelphia, Pa. It consists of a pipette to which is attached a capillary tube of 7.5 cm. in length and 0.106 cm. in diameter. Graduations A and B on the pipette define a volume of 13.2 c.c. Changes in surface tension are measured in terms of numbers of drops falling from the capillary outlet as the fluid contained in the pipette flows from graduations B to A.

In order to prevent plugging of the capillary tip with precipitated particles, the stalagmometer should be cleaned each day at the beginning and at the end of a series of analyses by drawing cleaning solution as high as the safety bulb above graduation B. After draining off the cleaning solution, first water is drawn into the apparatus in a similar manner and then an alcohol-ether mixture. If, in this process of cleaning, water enters the needle valve, this should be dried first by an alcohol-ether mixture and then by a current of air. Finally, the stalagmometer is dried by a current of air.

CALIBRATION OF THE STALAGMOMETER

For a given volume of water or bile, different stalagmometers may deliver varying numbers of drops. Hence, each stalagmometer should be calibrated in order to determine corrections to be applied in calculation of results. For this purpose, a stock solution is prepared containing 100 mg. of pure cholic acid in 100 c.c. of 0.01 NaOH. From this stock solution, serial dilutions are made up by adding each of the following aliquots, viz., 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 c.c. water in 100 c.c. volumetric flasks. These solutions are diluted to approximately 98 c.c. To each of these solutions, 6 N hydrochloric acid is added drop by drop with agitation until one drop, transferred by means of a stirring rod to a strip of filter paper saturated with tropeolin 00, shows a change from yellow to pink. After acidification, all solutions are diluted to 100 c.c.

Calibration of the stalagmometer should include first determinations of numbers of drops of distilled water falling from the stalagmometer during the

Received for publication, April 16, 1943.

flow of the water from the upper to the lower graduation. By means of the needle valve, the rate of fall of the drops must be adjusted to 30 per minute. Then readings are made of each of the diluted and acidified solutions of cholic acid prepared as described above.

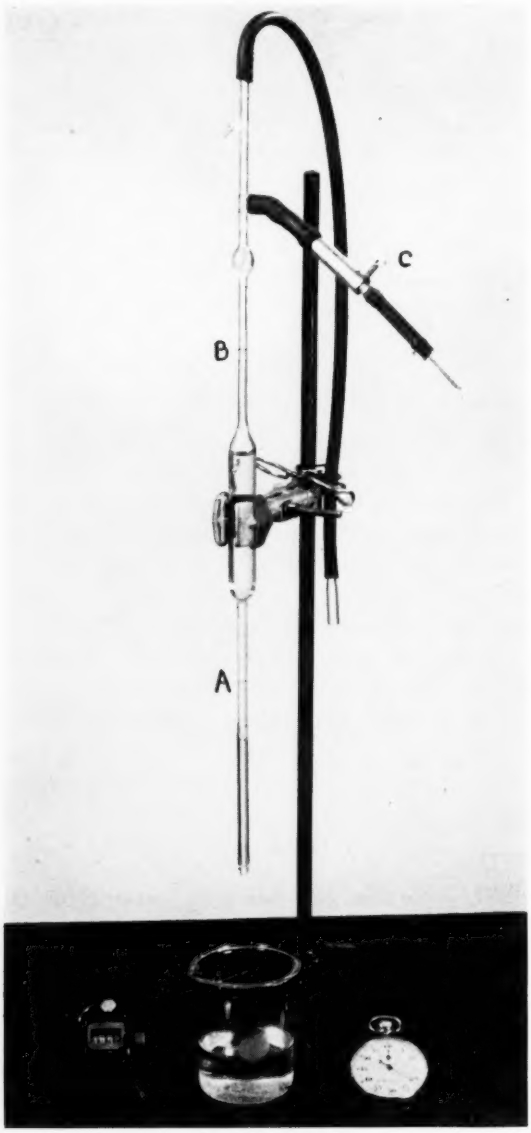

Fig. 1.

Concentrations of cholic acid in water corresponding to varying numbers of drops from one stalagmometer are shown in Table I. A similar table may be prepared for the particular stalagmometer being calibrated, or the readings may be corrected to conform with those in Table I. The procedure for determining corrections for a particular stalagmometer, A, is illustrated by the following example.

Before numbers of drops from stalagmometer A are referred to Table I for calculations of results, the numbers of drops must be multiplied by 0.94.

ANALYSES OF SPECIMENS OF BILE OR BILE FRACTIONS

Chart I shows the relationship between decrease in surface tension of water, as determined by the stalagmometer, and the concentration of cholic acid in solution. The maximum rate of rise of the curve lies within the range of concentrations from 2.0 to 5.2 mg. of cholic acid per 100 c.c. corresponding to stalagmometric readings from 157 to 190 drops. Within these levels, changes in concentration of cholic acid have greater effects upon the numbers of drops falling from the stalagmometer than do similar changes in other parts of the curve. In other words, this range represents the sensitive part of the curve. All readings should be maintained within the limits of 157 to 190 drops, as reported in Table I.

CHART I

CONCENTRATION OF CHOLIC ACID IN WATER MG. PER 100 C.C.	NUMBER OF DROPS FROM A	NUMBER OF DROPS TABLE I	RATIO $\frac{\text{DROPS—TABLE I}}{\text{DROPS—FROM A}}$
0	156	144	0.92
2.0	166	157	0.94
4.0	192	183	0.95
6.0	204	192	0.94
		Average =	0.94

An inspection of the bile fraction will suggest the proper range of dilution. Fluid specimens are diluted 10 or 25 times and viscid samples 50 to 100 or even 200 times.

A measured amount of bile (0.5 to 2.5 c.c.) is diluted with water to about 24 or 49 c.c. in a 25 or 50 c.c. glass stoppered volumetric cylinder. It is acidified with 6 N hydrochloric acid to the end point of the tropeolin 00 test paper using an outside drop test, and it is made up to the 25 or 50 c.c. volume mark.

TABLE I

TABLE FOR CALCULATION OF CONCENTRATIONS OF CHOLIC ACID FROM STALAGMOMETRIC READINGS

NUMBER OF DROPS	0	1	2	3	4	5	6	7	8	9
140					0	0.50	0.80	1.00	1.20	1.30
150	1.40	1.50	1.60	1.70	1.80	1.90	1.95	2.00	2.05	2.10
160	2.15	2.20	2.25	2.30	2.35	2.40	2.45	2.50	2.55	2.60
170	2.70	2.80	2.90	3.00	3.10	3.20	3.30	3.40	3.50	3.60
180	3.70	3.80	3.90	4.00	4.10	4.30	4.40	4.60	4.80	5.00
190	5.20	5.50	5.80	6.20	6.60	6.90	7.30	7.60	8.00	8.30

Values in the table give mg. per 100 c.c. of cholic acid.

The stalagmometer is filled by aspiration and allowed to drip into a 10 or 25 c.c. cylinder. The flow of drops is adjusted to 30 drops per minute or less. The drops are counted in the usual manner, but a reading is taken when the solution in the cylinder reaches a level of an even c.c. mark. Another reading is taken when the solution reaches the next c.c. level. The difference in readings represents the number of drops in 1 c.c. of solution. A reading can be taken on the next c.c. as a check. A previous calculation from the desired range

of readings (160 to 190 drops) and the volume of the stalagmometer (13.2 c.c.) will indicate when the number of drops per c.c. of the solution will give a total reading within the desired range.

$$\frac{160}{13.2} \text{ to } \frac{190}{13.2} \text{ is 12 to 14.4 drops per c.c.}$$

If the number of drops per c.c. is below the desired range, a fresh solution of higher concentration is prepared.

If the number of drops per c.c. is above the desired range, the solution is diluted 1:1 with adjustment of the pH to the specified end point. If the reading indicated more than a 1:1 dilution, it is advisable to prepare a fresh solution.

The stalagmometer is rinsed and filled with the new solution and the counting begun. If the preliminary test shows the reading is within the desired range, the stalagmometer reading of the full volume is taken.

As the solution in the stalagmometer flows out, the rate of flow diminishes to 20 drops a minute. It can be readjusted to 30 drops by a manipulation of the needle value without affecting the reading.

CALCULATION OF RESULTS

Stalagmometric readings for 2 or more dilutions of the bile, giving numbers of drops within a range of 160 to 190, are referred to a table prepared for that particular instrument; or readings corrected for the instrument, as described above, are referred to Table I. The concentration of cholic acid listed in the table corresponding to the reading is then multiplied by the dilution of the bile.

For example, a sample of bile diluted 1:100 gives, in duplicate determinations, 185 and 187 drops. The mean of 186 drops in Table I indicates a concentration of 4.4 mg. per 100 c.c. Hence, the concentration of bile salts as cholic acid in the bile fraction is $4.4 \times 100 = 440$ mg. per 100 c.c.

TABLE II

SUMMARY OF RECOVERIES OF CHOLIC ACID ADDED AS DESOXYCHOLIC ACID, CHOLIC ACID, OR BILE SALTS TO FRACTIONS OF BILE DRAINED FROM HUMAN SUBJECTS

SOURCE OF CHOLIC ACID ADDED TO BILE	RANGE OF CONCENTRATION OF CHOLIC ACID IN BILE MG. PER 100 C.C.	CHOLIC ACID ADDED MG. PER 100 C.C.	NO. OF EXPERIMENTS	AVERAGE RECOVERY PER CENT OF ADDED CHOLIC ACID	AVERAGE DEVIATION
Cholic Acid	103 – 138	25 – 140	7	104	±5
	360 – 480	75 – 500	6	99	±9
	615 – 990	240 – 960	9	108	±4
Desoxycholic Acid	300 – 390	38 – 380	12	96	±11
	570 – 700	60 – 600	7	97	±6
Bile Salts	103 – 107	47 – 100	3	104	±3
	300 – 480	42 – 500	13	98	±5
	615 – 990	32 – 960	18	100	±4
Averages			75	103	±7.3

PROBABLE ERROR OF THE METHOD

The accuracy of the method has been determined by a series of 75 recovery experiments summarized in Table II. The cholic acid concentrations of samples

of bile drained from human subjects were reinforced by the addition of either pure cholic acid, pure desoxycholic acid, or a commercial (Fairchild's) preparation of bile salts. Analyses of several samples of this commercial preparation of bile salts yielded an average of 34 per cent of cholic acid.

Quantities of cholic acid added to the bile samples gave theoretical increases varying from approximately 10 to 100 per cent above the original levels determined in these samples. Average recoveries of added cholic acid for the different levels of cholic acid concentration in the samples of bile varied from 96 to 108 per cent. For the 75 analyses, the average recovery was 103 per cent with an average deviation of ± 7 per cent.

SUMMARY

This report summarizes experiences over a period of three years with a simple, stalagometric method for determination of bile acid and salts in samples of bile drained by intubation of the duodenum.

In the description of the technique, particular emphasis has been placed upon conditions of dilution and acidification of samples of bile, and the range of stalagmometric readings which have yielded most satisfactory results.

A series of 75 experiments have given an average recovery of 103 ± 7.3 per cent of cholic acid, added either as cholic acid, desoxycholic acid, or a mixture of bile salts to samples of bile drained from human subjects.

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THE SEPARATE DETERMINATION OF THE FATTY ACID FRACTION AND OF THE NEUTRAL FAT PLUS STEROL FRACTION IN FECES*

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THE separate determination of fatty acids and of the neutral fat portion in feces theoretically offers valuable information as to the efficiency of fat digestion and absorption. Analytical procedures have been described by a number of investigators, e.g., Tidwell and Holt,[1] Fowweather,[2] Holt, Courtney, and Fales,[3] and Saxon,[4] but all the procedures are quite long and require considerable manipulation. Several years ago a relatively simple method for the determination of neutral fat and cholesterol in animal tissue was described by Outhouse and Forbes.[5] Since a simple method for the determination of fat and free fatty acids should be of considerable value in clinical chemical lab-

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Received for publication, May 3, 1943.

oratories, it was decided to determine whether this method could be modified in order to allow the independent determination of the neutral fat fraction and fatty acids in a mixture of the two. After attempting various procedures, a method was finally worked out and applied with satisfactory results to fecal analyses. No distinction is made between fatty acids present as soaps or as free fatty acids. Sterols are included in the neutral fat fraction. Bloor's oxidative technique[6] with minor modifications was used throughout.

Determination of Neutral Fat Plus Sterols.—Approximately 3 Gm. of feces are air-dried, as recommended by Tidwell and Holt,[1] ground thoroughly, and last traces of moisture removed in a desiccator. About 0.3 Gm., accurately weighed, is ground in a mortar with 5 Gm. of doucil,* an artificial zeolite, and transferred quantitatively to a 125 c.c. glass-stoppered Pyrex bottle. 100 c.c. of chloroform are added, followed by 0.8 c.c. of 10 per cent sodium hydroxide to precipitate the free fatty acids as their sodium soaps. The bottle is shaken well and set aside with occasional shaking for several hours and preferably left overnight. The chloroform solution is filtered through a fat-free rapid filter paper, and 2 to 5 c.c., depending upon the apparent amount of fat present in the sample, are transferred to a 125 c.c. glass-stoppered Pyrex Erlenmeyer flask. The flask is placed in warm water, and the solvent is completely removed with the aid of a stream of air played upon the surface of the liquid. Five c.c. of a silver chromate solution in sulfuric acid are added, and this is followed by 3 c.c. of 1 N potassium dichromate. The flask is stoppered loosely, the solution gently mixed, and the flask put in an electric oven at 95° C. The flasks are removed after about five minutes, rotated to stir up the contents, stoppered tightly, and replaced in the oven for two hours. Control flasks using the same amount of silver chromate and potassium dichromate solutions are run at the same time. The stoppers are removed immediately after the flasks are taken from the oven, and shortly thereafter approximately 60 c.c. of cold distilled water are added. Ten c.c. of 10 per cent potassium iodide are added without stirring, and the liberated iodine is titrated without delay with 0.1 N sodium thiosulfate, using 1 per cent starch solution as indicator. A small granule of sodium hydroxide should be added to the potassium iodide solution to prevent decomposition. The starch solution should not be added until the yellow color of the iodine has practically disappeared. The difference between the titration value of the control and unknown solution is a measure of the lipids present in the unknown sample taken for analysis. A value of 3.6 c.c. of 0.1 N sodium thiosulfate is taken as equivalent to 1 mg. of neutral fat. Sterols are extracted along with the neutral fat and appear in the calculations as neutral fat. Phospholipids do not interfere as they are removed by the doucil.

SPECIAL REAGENTS REQUIRED

Silver Chromate Reagent.—To 5 Gm. of silver nitrate dissolved in 25 c.c. of water are added 5 Gm. of potassium dichromate dissolved in 50 c.c. of water. The precipitated silver dichromate is separated by centrifugation, washed twice with water by centrifugation, and then dissolved without drying in 500 c.c. of pure concentrated sulfuric acid.

*The doucil was obtained from W. A. Taylor and Company, Baltimore, Maryland.

Sodium Thiosulfate Standard (0.1 N) Solution.—Weigh out 25 Gm. of ordinary C.P. sodium thiosulfate or 24.83 Gm. of the pure dry recrystallized salt. Dissolve in water and dilute to a liter. Boiled distilled water must be used. This solution is standardized against acid potassium iodate. Weigh accurately 0.3249 Gm. of acid potassium iodate. Dissolve in 50 c.c. of water, heating gently if necessary. Transfer the solution to a 100 c.c. flask, rinsing the beaker carefully, and dilute to volume with water. This solution is exactly decinormal. Pipette 25 c.c. into an Erlenmeyer flask, add 1 gram of potassium iodide dissolved in a little water and a few cubic centimeters of dilute hydrochloric acid. Titrate immediately with the thiosulfate solution, using starch as indicator.

Determination of Soaps Plus Free Fatty Acids.—A weighed amount, 0.1 to 0.2 Gm., of the thoroughly ground sample is transferred to a 125 c.c. Pyrex glass-stoppered bottle and 4 c.c. of 1:4 sulfuric acid added (1 vol. of sulfuric acid plus 3 vols. of water). The bottle is warmed gently on a hot plate so as to convert all soaps to free fatty acids. This heating should be continued until the solution is close to the boiling point and considerable water has condensed on the sides. The flask is allowed to cool and 5 Gm. of doucil added. After mixing, 100 c.c. of chloroform are added, and the bottle is shaken thoroughly, then is set aside with occasional shaking for several hours, and preferably is left overnight. The chloroform solution is filtered through a fat-free rapid filter paper, 2 to 5 c.c. of the filtrate are evaporated to dryness in an oxidation flask, and the total lipid content is determined by the oxidation method already described. Since the oxidation value of the fatty acids approximates that of the corresponding fat, 3.6 c.c. of 0.1 N sodium thiosulfate has been taken as equivalent to 1 mg. of lipid material, whether present as neutral fat or fatty acids. The difference between the lipid content per 100 Gm. as determined above and that obtained for the neutral fat portion gives the fatty acids present as soaps or as free fatty acids.

TABLE I

RECOVERY OF NEUTRAL FAT AND FREE FATTY ACIDS FROM MIXED SOLUTIONS

EXPERIMENT NUMBER	LIPIDS PRESENT			LIPIDS RECOVERED				
	TOTAL GM.	NEUTRAL FAT GM.	FATTY ACID GM.	TOTAL GM.	NEUTRAL FAT OBTAINED GM.	NEUTRAL FAT CORRECTED GM.	FATTY ACID OBTAINED GM.	FATTY ACID CORRECTED GM.
1	.1391	.0577	.0814	.1358	.0614	.0594	.0744	.0764
2	.1451	.0445	.1006	.1363	.0470	.0450	.0893	.0913
3	.0955	.0417	.0538	.0952	.0409	.0389	.0543	.0563
4	.1086	.0502	.0584	.1068	.0520	.0500	.0548	.0568
5	.1086	.0502	.0584	.1075	.0544	.0524	.0531	.0551
6	.1829	.1056	.0773	.1812	.1130	.1110	.0682	.0702
7	.1531	.0773	.0758	.1481	.0812	.0792	.0669	.0689
8	.1147	.0630	.0517	.1140	.0622	.0642	.0518	.0538
9	.2109	.0902	.1207	.2092	.0992	.0972	.1100	.1128
Total	1.2585	.5804	.6781	1.2341	.6113	.5973	.6228	.6408
			Per cent error	−1.1	+5.3	+2.9	−8.1	−5.5

Before applying the method to fecal analyses, it was necessary to determine the recovery from known solutions of fat and fatty acid. In these experiments a weighed amount of recrystallized tripalmitin and stearic acid was dissolved in 200 c.c. of chloroform. Approximately half of the solution was transferred to a 125 c.c. glass-stoppered bottle containing 5 Gm. of doucil, after which

0.8 c.c. of 10 per cent sodium hydroxide was added and the solution thoroughly shaken. The remainder was transferred to a similar bottle containing 5 Gm. of doucil plus 4 c.c. of 1:4 sulfuric acid. Both bottles were thoroughly shaken and allowed to stand with occasional shaking for several hours, and usually left overnight. They were then filtered and analyzed as previously described, and the amount present in the original 200 c.c. of solution was calculated. In the calculations 3.60 c.c. of 0.1 N sodium thiosulfate have been taken as equivalent to 1 mg. of tripalmitin and 3.66 c.c. for 1 mg. of stearic acid. The experimental results are shown in Table I. The values for neutral fat are on the average approximately 5 per cent too high. This is due primarily to a slight solution of the fatty acids in the chloroform, even in the alkaline medium. Experiments with pure stearic acid under the condition used showed an average solubility of approximately 1 mg. per 100 c.c. of chloroform. If corrections are made for this solubility, the per cent recovery is quite satisfactory. In the usual fecal analysis an error of this magnitude can be neglected. This has been done in obtaining the experimental results recorded in Table II.

TABLE II

LIPID ANALYSIS OF NORMAL FECES

EXPERIMENT NUMBER	NEUTRAL FAT PLUS STEROLS	FATTY ACIDS PLUS SOAPS
	Per cent	Per cent
1	8.3	10.9
2	6.5	8.2
3	10.0	2.9
4	7.5	9.3
5	6.7	10.9
6	8.7	7.1
7	8.0	8.9

All values are given in per cent dry weight.

Experiments with calcium stearate following the procedure described for soaps and free fatty acids gave satisfactory recovery of the stearic acid. The analytical results obtained on normal feces and recorded in Table II are in good agreement with the commonly accepted normal values. Although approximately 0.3 Gm. is very suitable for normal feces, the sample should be reduced to about half this value if the fat content is apparently high.

SUMMARY

A relatively simple method for the determination in feces of neutral fat plus sterols, free fatty acids, and soaps is described.

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A SIMPLE AND ACCURATE METHOD FOR THE DETERMINATION OF MERCURY IN BIOLOGIC MATERIAL*

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THE use of dithizone as a colorimetric reagent for mercury is due to Fischer and Leopoldi,[1] and Fischer,[2] and has recently been adapted to use in biologic materials by Laug and Nelson.[3]

The colorimetric method is accurate enough to supplant tedious older methods, which depend upon electrolysis or amalgamation of the metallic element for its ultimate determination, or upon titration methods involving iodide or thiocyanate. The present method has the advantage of extreme simplicity, speed, and a broad range. A single analysis should not take longer than five minutes, after digestion of organic material is completed.

DISCUSSION

The investigators cited above have regarded copper as an interfering element. They separated copper from mercury at pH 1 by the use of KI, KBr, or $Na_2S_2O_3$. Finally, the mercury dithizonate is formed at pH 6. Both of these steps are eliminated in the present method. The number of operattions in the procedure are thereby considerably reduced. Formation of the mercury dithizonate at pH 1 further eliminates the problem of maintaining lead free reagents and apparatus. At pH 6 dithizone is extremely sensitive to lead, and in the earlier methods special consideration of this was required.

We have found that copper need not interfere, if the proper conditions of pH are maintained. In acid solution, dithizone reacts with gold, palladium, platinum, mercury, silver, copper, and bismuth. The latter do not, however, react at pH 0 to 1.0. In 50 c.c. 0.25 N HCl, 1,000 mg. of copper does not change the color of dithizone reagent. Silver does not react in the presence of excess halogens. Mercury and the noble metals alone, therefore, react with dithizone under these conditions, and the latter will rarely interfere with analysis of biologic materials. The yellow color of the dithizone-mercury complex may then be regarded as specific in the presence of 0.25 N HCl and in the absence of the noble metals. The presence of 5 to 7 c.c. sulfuric acid (from the organic digestion) brings the pH to 0.2 and does not alter the above considerations in any way.

Fig. 1 shows that at 490 mμ conditions are proper for a "mixed color" determination of mercury with a spectrophotometer. Here the green dithizone solution transmits rather freely, and the yellow mercury complex has its absorption maximum. Solutions to be measured will range from green through light green and dark yellow to light yellow. The standard or unknown is measured against the dithizone solution. Beer's law is obeyed; the optical density has a linear relation to the concentration of mercury.

In the digestion of organs and urine, it was not found necessary to connect a water-cooled condenser to the digestion flask. A mixture of equal weights of sulfuric and nitric acids was used. It was found that the digestion proceeds

*From the Wallace Laboratories, Inc.
Received for publication, May 28, 1943.

smoothly if about three times as much mixed acid as organic material is used. Occasional charring may occur; the addition of small supplementary amounts of nitric acid will obviate this. The digestion is carried out in a micro-Kjeldahl flask with ground glass joint. The digestion may proceed with an air-cooled condenser rather than under reflux. We have found that elimination of the water-cooled condenser considerably decreases the time of digestion and no loss of mercury is involved. Table I shows recovery of mercury using the method described below. Table II shows noninterference of copper over a wide range.

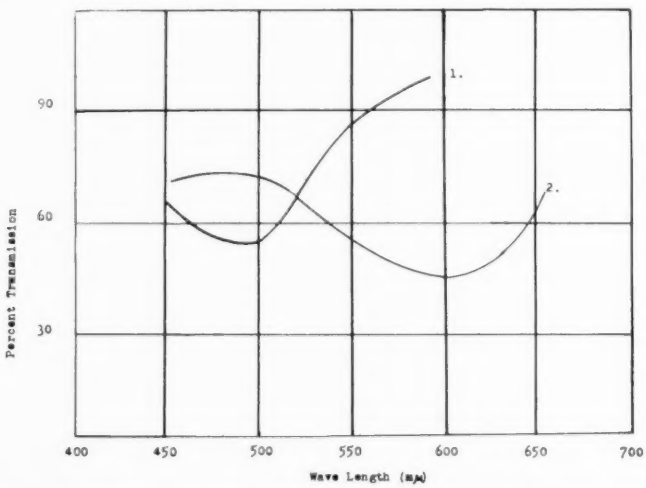

Fig. 1.—1. Mercury-dithizone complex ($CHCl_3$ solution). 2. Dithizone (in $CHCl_3$). (Readings from Coleman spectrophotometer. Solutions shaken with .25 N HCl in all cases.)

REAGENTS

1. Sulfuric Acid. Analytical Reagent.

2. Nitric Acid. Analytical Reagent. An equal weight mixture of 1 and 2 was used as the stock digestion solution.

3. Hydroxylamine hydrochloride chemically pure. A 20 per cent aqueous solution is used to destroy any excess nitric acid.

4. 0.25 N HCl. From chemically pure HCl.

5. Dithizone solution. 10 mg. dithizone (Eastman) dissolved in 1 liter of chloroform. This is a light green solution. (Transmission curve shown in line 2 of Fig. 1.) 10 c.c. will be entirely converted to the metal salt by 25 micrograms of mercury. The resulting yellow solution is plotted in line 1 of Fig. 1.

PROCEDURE

To about 5 Gm. of organic material add 10 to 15 c.c. of acid digestion mixture in a long-necked Kjeldahl flask, to which is fitted (using a glass joint) an air condenser about 18 to 20 inches long. Digestion begins spontaneously. After ten minutes apply a gentle flame, and increase the heat gradually until the full flame of the burner is used. After an hour the nitric acid fumes disappear and the digestion is finished.

The ash solution is cooled and poured into a separatory funnel containing 25 c.c. of .25 N HCl and 2 c.c. of 20 per cent hydroxylamine hydrochloride. Add 10 c.c. of dithizone reagent solution, shake 50 times, and collect the $CHCl_3$

TABLE I

SUBSTANCE	AMOUNT	MICROGRAMS HG ADDED	TYPE OF CONDENSER	MICROGRAMS HG FOUND (RECOVERY)
Rabbit blood	1 c.c.	10	Water-cooled	9.8
Rabbit blood	5 c.c.	10	Water-cooled	9.9
Rabbit blood	1 c.c.	10	Air-cooled	9.8
Rabbit blood	1 c.c.	10	Air-cooled	9.7
Guinea pig liver	4 Gm.	10	Air-cooled	10.0
Guinea pig liver	3 Gm.	5	Air-cooled	4.9
Human urine	20 c.c.	20	Air-cooled	20.2
Human urine	20 c.c.	20	Air-cooled	20.0

TABLE II

SUBSTANCE	AMOUNT	MICROGRAMS HG ADDED	MICROGRAMS CU. ADDED	TYPE OF CONDENSER	MICROGRAMS HG FOUND (RECOVERY)
Rabbit blood	5 c.c.	5	0	Air-cooled	4.9
Rabbit blood	5 c.c.	5	5	Air-cooled	5.0
Rabbit blood	5 c.c.	5	15	Air-cooled	5.0
Human urine	5 c.c.	5	50	Air-cooled	4.8
Human urine	5 c.c.	5	100	Air-cooled	4.9
Rabbit blood	5 c.c.	5	100	Air-cooled	5.1

layer. The mercury dithizone thus formed is very sensitive to light and should not be exposed to bright sunlight. The extract is measured with the spectrophotometer against dithizone at 490 mμ, and the reading is compared with that on a standard curve.

This calibration curve, conforming to Beer's law, sets optical density against concentration of mercury. The standards may be prepared by adding known amounts of mercury (not exceeding 25 mg.) to 25 c.c. of 0.25 N HCl, and extracting, as above, with 10 c.c. dithizone. The optical density of the extract is measured against that of dithizone solution at 490 mμ. We have not found it necessary to include the other reagents used in the digestion in this standardization. They have no effect on the final color. Twenty-five milligrams of mercury will convert all the dithizone to the yellow mercury complex. Larger amounts therefore must be determined by taking aliquot samples of the unknown after digestion.

An alternate procedure, which is accurate enough for general biologic work, may be employed in the absence of a spectrophotometer. In this method the mercury is titrated with dithizone solution. For the concentration of dithizone used, 1 c.c. of the solution is equivalent to 2.5 mg. of mercury. Small portions of dithizone are added to the funnel, which contains, as above, the mercury sample 0.25 N HCl and hydroxylamine hydrochloride. The end point is reached when the dithizone in the funnel is olive green, indicating slight excess of unreacted reagent. The number of micrograms of mercury in the sample is equal to the cubic centimeters of dithizone solution x 2.5. It is advisable, however, to standardize the dithizone solution by actual titration against a known amount of mercury.

The titration method is also useful when over 25 mg. of mercury are present. In this case the spectrophotometer cannot be used, for 10 c.c. of dithizone reagent is completely converted to the mercury salt, and a "mixed color" determination is impossible. Successive additions of dithizone, however, will yield the green and point color. The total amount of dithizone added will then be a measure of the mercury present.

SUMMARY

A rapid, sensitive, colorimetric method for analysis of mercury, particularly in biologic materials, has been presented. Dithizone in $CHCl_3$ reacts with mercury at pH 1 (only gold palladium and platinum would interfere) to form a yellow complex which may be analyzed by the "mixed color method" in the spectrophotometer, or may be used as the basis for direct titration of the mercury.

The author wishes to thank Miss Blanche Kohut for her technical assistance.

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DETERMINATION OF DIODRAST-IODINE IN URINE AND IN FILTRATES OF PLASMA*

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THE accurate determination of diodrast-iodine has become widely practiced in the determination of diodrast clearance and of functioning tubular secretory mass (Smith, Goldring, and Chasis,[1]). The following simple procedure has proved very satisfactory in daily use during the past two years. The method was orginally developed from Alpert's procedure[2] as a titrimetric procedure in which diodrast-iodine was determined as iodate by thiosulfate titration. The colorimetric procedure used by Flox, Pitesky, and Alving[3] was then applied to it without modifying other aspects of our method.†

Principle.—"Duponol PC"‡ is used to stabilize recovery of diodrast from plasma filtrates. Diodrast-iodine is oxidized by treatment with bromine water in acid solution. The excess of bromine is removed by addition of sodium formate, and the iodate iodine (Flox, Pitesky, and Alving,[3] 1942) content is determined photocolorimetrically.

Preparation of Reagents.—1. Acid $ZnSO_4$ with Duponol PC. 27 Gm. $ZnSO_4$ and 30 c.c. of approximately 1 N H_2SO_4 are made up to about 1 liter with water. One gram of Duponol PC is added and put in solution with the least possible shaking. Ten c.c. of the mixture in about threefold dilution are titrated with 0.15 N NaOH, using phenolphthalein as indicator. The pink color of the end point should persist for one minute. From the value obtained in the titration, the acid $ZnSO_4$ solution is diluted until 10 c.c. of it exactly neutralizes 11.2 c.c. of 0.15 N. NaOH.

2. 0.15 N NaOH. The solution is prepared by dilution of "carbonate-free" NaOH in recently boiled distilled water. It is protected from atmospheric CO_2 by a soda-lime trap. Plasma filtrates prepared as directed below should be of pH 6.7 to 6.8. The recovery of diodrast from plasma becomes less complete as

*From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital.

†Details of this procedure were furnished us before its publication through the kindness of Dr. Alving.

‡E. I. Du Pont and Company, Wilmington, Del.

Received for publication, May 31, 1943.

the pH range 6.0 to 6.2 is reached, while the plasma "blank" increases rapidly about pH 6.8. The accuracy of preparation of reagents should therefore be checked by the determination of pH in plasma filtrate before the solutions are placed in use. The addition of Duponol PC increased the pH range over which recovery of diodrast-iodine from plasma is possible and permits full recovery at plasma dilutions of less than 1:15.

3. Bromine water. An excess of liquid bromine is added to 500 c.c. of distilled water. The mixture is stored in the refrigerator, water and bromine being replenished as required.

4. Sodium formate. 10 Gm. of reagent grade sodium formate are dissolved in 100 c.c. of water. The solution is kept in the refrigerator and made freshly each week.

5. Alkaline KI. 50 Gm. of KI, reagent grade, are dissolved in approximately 100 c.c. of 0.01 N NaOH. The solution is protected from light by storage in an amber dropping bottle.

PROCEDURE

1. Precipitation of plasma proteins: A modification of the Somogyi zinc precipitation is used. To one volume of heparinized plasma are added five volumes of acid $ZnSO_4$ with Duponol. The mixture is allowed to stand for about thirty minutes. Five volumes of 0.15 N NaOH are then added dropwise with constant agitation. The mixture is filtered at once through a No. 1 Whatman paper *without* prior centrifugation.

Because of the high dilutions (1:1000) used, comparable precipitation of protein from urine samples is unnecessary except in the presence of gross proteinuria or hematuria. In such cases, 0.5 c.c. each of the $ZnSO_4$ and NaoH solutions are added to the 1 or 2 c.c. samples of urine in a volumetric flask, the mixture then made up to volume with water and a sufficient amount filtered.

2. Oxidation of Diodrast-Iodine to Iodate: 0.2 c.c. of 85 per cent $H3PO_4$ is added to 10 c.c. of plasma filtrate or diluted urine in a colorimeter absorption tube. One c.c. of bromine water is added and the mixture shaken until homogenous. The tube is then placed in a boiling water bath for three minutes, when it is rapidly cooled to room temperature in an ice bath. The wall of the tube is then carefully dried by wiping with a soft towel, and 1 c.c. of 10 per cent sodium formate added. The solutions are mixed by shaking and then allowed to stand 20 to 30 minutes at room temperature.

3. Measurement of Color Density: The clarity of the mixture before addition of KI permits determination of the individual "center-settings" of each tube, thus correcting for inaccuracies in the calibration of individual tubes and permitting the use of tubes which have become too scratched or worn for other uses. The "center-setting" is determined for each tube in the series, using the appropriate color filters. One drop of alkaline KI is then added, the "center-setting" is appropriately adjusted, and the reading is made at exactly two minutes.

Light filter No. 400 is suitable for the determination of plasma "blank" and of plasma diodrast-iodine concentration during clearance periods. Filter No. 440 is ordinarily used for the determination of diodrast-iodine in urine dilutions and No. 490 for the concentrations in plasma and urine which obtain in determination of Tm_D.

Calculation.—The determination of diodrast-iodine concentration from

galvanometer readings is made by reference to a calibration curve from the preparation either of iodate solutions or from dilutions of 35 per cent diodrast solution treated with all reagents exactly as described. The separately measured plasma "blank" should be subtracted from the observed plasma concentration, and the result multiplied by the dilution factor (11); thus (0.175 to 0.002) 11.0 = 1.925 mg. per 100 c.c. of plasma, where 0.0175 is the apparent diodrast-iodine content of the 10 c.c. of unknown plasma filtrate and 0.002 the value of the plasma "blank."

The high dilutions of urine used obviate any appreciable error from urinary "blank."

Results.—The mean recovery in 34 determinations of diodrast-iodine added to human plasma was 100 per cent. The range of recovery in this series varied from 97.5 to 102 per cent. The concentrations used ranged from 1.9 to 66 mg. per 100 c.c. of plasma.

We found the method particularly useful because: (a) There is the convenience and safety of bromine water and sodium formate in contrast, respectively, with bromine and phenol reagents; (b) the separate determination of "center-setting" for each tube obviates errors due to differences between individual tubes, such as result from etching or scratches and permits the use of tubes, which would otherwise be discarded; and (c) the use of 10 c.c. of plasma filtrate in a final volume of 12.2 c.c. yields a color density from 1.0 mg. per 100 c.c. plasma diodrast-iodine which, using filter No. 400, reaches at about the center of the galvanometer. The use of a special filter for determination of low concentrations of iodate-iodine is thus avoided.

The use of "Duponol PC" was practiced because it was found that its detergent action made it possible to obtain full recovery of plasma diodrast in zinc filtrates in which the plasma had been diluted only 10 or 11 times, whereas only 90 to 95 per cent was recovered in these filtrates in the absence of "Duponol PC," when it was added in large excess or when a series of other detergents were tried. Full recovery of diodrast-iodine, however, may be obtained from plasma in zinc filtrates without the use of the detergent when the plasma is 15 times diluted.[3] We have found the more concentrated filtrates advantageous, and therefore retain the use of "Duponol PC." Probably some other application in clinical chemistry of this action of detergents will be found.

SUMMARY

A convenient method is described for determination of diodrast-iodine in urine dilutions or plasma filtrates. The method depends upon the detergent action of "Duponol PC" for full extraction of diodrast from plasma; the diodrast-iodine of the plasma filtrates is then oxidized with bromine water and the excess of bromine decomposed by adding sodium formate. The iodate-iodine content of the samples is determined photocolorimetrically by the method of Flox, Pitesky, and Alving.[2]

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MEDICAL ILLUSTRATION

COLORING AND APPLYING FACIAL PROSTHESES*

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IN CONSIDERING the color result of a final prosthesis it must be realized that there are seven distinct steps that can be used in color work alone to obtain the finished product. All of these are not necessary in every instance, but each step is worthy of consideration. The procedure begins with the latex mix and does not end until cosmetics are applied to the prosthesis when it is in place on the patient. The steps taken to produce the color effect are as follows:

1. Counteracting the natural yellow color of rubber and its tendency to darken.
2. The addition of water-soluble pigments and dyes to the latex mix to produce a basic monochrome skin color suitable to the individual patient.
3. Blended color effects on the dried rubber prosthesis achieved with oil-soluble dyes.
4. Detail effects produced with alcohol-soluble dyes.
5. Placing of hair, glass eyes, and fingernails in the rubber cast.
6. Highlighting or lacquering the surface.
7. The application of cosmetics.

These steps are of sufficient importance to require a detailed description of each.

1. *Counteracting the Natural Yellow Color of Rubber.*—Unfilled rubber turns dark on prolonged exposure to sunlight. It assumes a yellow cast which varies from light cream to raw umber. Steps to forestall this change must be taken while the rubber is in the latex state. Zinc oxide (a thickening agent) and clay or chemical fillers should be added. The zinc oxide should not exceed 2 per cent in vulcanized latex or 4 per cent in unvulcanized latex; otherwise the final prosthesis may crack. This cracking can occur even when the prosthesis is soft and pliable. An excess of zinc oxide would cause the prosthesis to become so opaque that it would not possess the translucency of normal Caucasian skin.

In addition to the zinc oxide, clay fillers can be added to prevent the rubber from becoming too dark. The clay fillers should not exceed 10 per cent or the translucency will be lost. In fact, if the rubber contains both zinc oxide (2 per cent) and clay filler (10 per cent), the prosthesis may lack the translucency of normal Caucasian skin. The proportions should invariably be less than the percentages mentioned. The mixture is balanced to suit specific types of skins. It is often better to make up stock solutions of the rubber

*From the Department of Moulage and Prosthetics, Army Medical Museum.
Received for publication, July 9, 1943.

latex containing different percentages of clay filler. I believe the clay fillers are more suitable than zinc oxide for this purpose. If clay is used various stock solutions of latex can be prepared, each having the proper basic colors incorporated, and each being slightly different from the other. A dried sample from each lot may be attached to the neck of the bottle. These samples are placed next to the patient's skin to determine which solution is most suitable

Fig. 1.—The front view of a patient requiring a prosthesis.

for making the final prosthesis. If zinc oxide is used instead of clay to prevent darkening, the stock solution may in time coagulate in the bottle and become unfit for further use. When zinc oxide is chosen, it must be incorporated as a thin water paste into the latex just before it is poured into the mold. Stock solutions containing a small amount of zinc oxide that are kept for long periods of time become so thick that it is difficult to pour them into

molds having small openings. If the stock solution is diluted with water, it continues to sink in the mold because the plaster absorbs the water, and air pockets will likely form in the cast. These may be corrected by refilling the mold as the solution sinks in the opening. Some stock solutions containing zinc oxide thicken considerably in twenty-four hours. Although this may be an advantage when it is necessary to build up a coagulum deposit, as on agar molds of hands, it is a disadvantage in molds that have small openings as in a prosthesis of the ear or nose.

Fig. 2.—The prosthetic nose in place.

It seldom takes more than 100 c.c. of latex mixture to make the average prosthesis of an ear or nose. Therefore, the 2 to 4 per cent of zinc oxide required for this quantity can be added with an eye dropper from a stock

solution of zinc oxide ground in water to a thin paste or viscous liquid. For example, there are about 15 drops to 1 c.c.; therefore, 30 drops or 2 c.c. of this water dispersion can be added to 100 c.c. of the rubber mixture to get the proper mix for a prosthesis. This is only a rough method of figuring but it is sufficiently accurate for the purpose. If the rubber mixture contains the clay filler, the zinc oxide content can be reduced or eliminated.

Fig. 3.—The side view of the same patient without the prosthesis.

2. *The Monochrome Base Color.*—Usually a monochrome base color is added to the latex mix to give it a general tint similar to that of the patient who is to wear the prosthetic appliance. As noted before, it is customary to have on hand various stock solutions of slightly different flesh colors because each patient's skin tones vary. This is particularly true among Negroes. As a

rule, six stock solutions suffice for Caucasians. Eight should be enough for Negroes. When a skin tone is between the color of two stock solutions, the proper color may be made by mixing the two. A dried sample of each skin tone should be attached to the neck of each bottle of stock solution, since the dried latex is different in color from the liquid latex. It is invariably darker. These samples are placed next to the skin to match the color. The prosthetist generally chooses one a shade lighter than the patient's color because other color mediums are added which eventually create the desired effect. Furthermore, all rubber prostheses tend to darken on prolonged exposure to sunlight. From time to time, over a period of months and years, it may become necessary to remake a prosthesis, and for this reason the mold of each patient should be filed away for future use.

To obtain the stock monochrome solutions previously mentioned, color in the form of pigments or dyes is added to the latex filler mixture. These pigments and dyes must be confined to certain restrictions. For example, they should not have an acid reaction to litmus paper. If they do, they will coagulate the latex into a mass or into isolated particles. They should contain no copper. If copper is present, even in small quantities, resinification of the rubber will follow. If opaque pigments are used to excess, the translucency is destroyed in the resulting prosthesis. Translucency is essential to naturalness, and for this reason transparent dyes are usually employed. Unfortunately, the average dyes available for rubber are not as permanent as pigments. Pigments are ground with water before being incorporated. Dyes are added to water until translucent solutions without any sediment are formed before incorporation. A considerably larger quantity of pigments than dyes is necessary to obtain the desired shade. When adding both pigment and dye solutions to small quantities of latex, it is advisable to use an eye dropper. The color is added a drop at a time while stirring the mixture.

In considering the brownish-red tones of Negroid skin, one may say that all three primary colors are often employed to obtain the desired effect. For this reason pigments and dyes of various shades are listed.

The following color ingredients are suitable for latex mixes:

Lithopone
Sulfide of antimony (if free from calcium sulfate)
Inactive carbon or lamp blacks, ivory black or the like
Green chromium oxide
Natural red iron oxide
Organic colors, if available in powdered or paste form

Among the water-soluble colors manufactured by E I Du Pont de Nemours & Company are the following: Pontamine fast dyes which are compatible with rubber latex and are to a great degree permanent.

Pink BL
Brown 2 RL
Blue 4 GLN
Red 8 BNL
Blue SRL
Gray BL
Orange 2 GL
Yellow NNL

These dyes are in powder form. About 20 Gm. of the powder are added to 200 c.c. of water. This mixture is added drop by drop to small quantities of rubber. The concentration of dye in water can be stronger for Negroid skin. It is difficult to give specific amounts of dye to be added to water because each person's skin varies in color, and the concentration of dye to water may vary. The prosthetist must depend on his sense of color to determine the amount of dye to be added to each latex mix or to a stock solution.

Fig. 4.—A side view of the same patient with the nose in place.

Most dye manufacturers list dyes that are suitable for incorporating with rubber. These manufacturers are listed in the Chemical Buyers' Guide Book,* which is practically indispensable to the prosthetist.

*Chemical Buyers' Guide Book, Chemical Industries, 522 Fifth Avenue, New York City.

In any event, the monochrome tint applied to the latex mix is kept somewhat lighter than is desired for the finished prosthesis, because additional oil-soluble color will be added later.

After the desired tint is chosen the mold is poured; the cast is dried in the mold and removed. The seam lines are trimmed with instruments heated over a Bunsen burner. Often a syruplike substance is thus produced. This is burnt resinified rubber and may be removed with a small piece of cloth dampened with turpentine. In fact, the prosthesis can be given a rather vigorous cleaning with the turpentine cloth. It serves to remove the tiny imperfections caused by the smallest air bubbles in the mold. The larger positive impressions of air bubbles must be cut away. The turpentine may cause the rubber cast to swell, but this will rectify as the cast dries. When one really masters the use of the tools and a knowledge of the temperature at which to use them, seam lines may be cut away with the hot tool without resinifying the rubber. Moreover, skin detail can be replaced and defects in casting removed or repaired without damage to the surface. Although depressions caused by faulty casting can be modeled out by the addition of small amounts of latex to the cast, this procedure is not always successful. It is generally better to remake the cast. The rubber cast should be washed thoroughly in soap and water after it is cleaned with turpentine.

When the prosthesis has been trimmed carefully and all defects have been corrected, it is time to use the oil-soluble dyes.

3. *Blended Shading With Oil-Soluble Dyes.*—Unvulcanized rubber is soluble in carbon disulfide with 5 per cent absolute alcohol, benzene (benzol), chloroform, benzine and oil of turpentine. Vulcanized rubber, however, only swells in these solvents, and after prolonged soaking it loses its strength to a point where it crumbles readily. Benzine being more volatile than turpentine has a quicker swelling action on the vulcanized rubber than turpentine. If oil-soluble dyes are mixed with these rubber solvents, a coloring medium is produced that will sink readily into the rubber and give permanent tints. It should be realized, however, that the dye continues to bleed into the rubber and becomes less intense. By means of this change the prosthetist is able to obtain more lifelike blended color effects on a monochrome prosthesis. In other words, skin is not a monochrome, but is made up of delicately blended areas of different shades of the same color or different colors. The same effect cannot be obtained with pigments because these are composed of microscopic particles of opaque matter which do not sink or bleed into the rubber but lie on the surface. Even if perfect blending with pigments were possible, the color would in a short time wash from the surface of the prosthesis and expose the monochrome-tinted rubber.

An oil-soluble dye applied in the same manner sinks into the rubber, and the prosthesis may be washed or even scrubbed immediately afterward. As time progresses the dye blends or bleeds further into the rubber and becomes more uniformly distributed or thoroughly blended with other shades. For this reason the prosthesis is generally colored more deeply than is desired for immediate use. The complete blending may require a few days. After several experiments, however, the worker can anticipate the effect that time will produce. It is assumed that once the solvent has evaporated from the rubber the blending will cease.

The difference between pigments and dyes can readily be detected with a microscope. For example, samples of a red pigment and a red dye are placed on a microscopic slide and studied through transmitted light. The pigments will appear as black specks and the dye will still appear red on the slide.

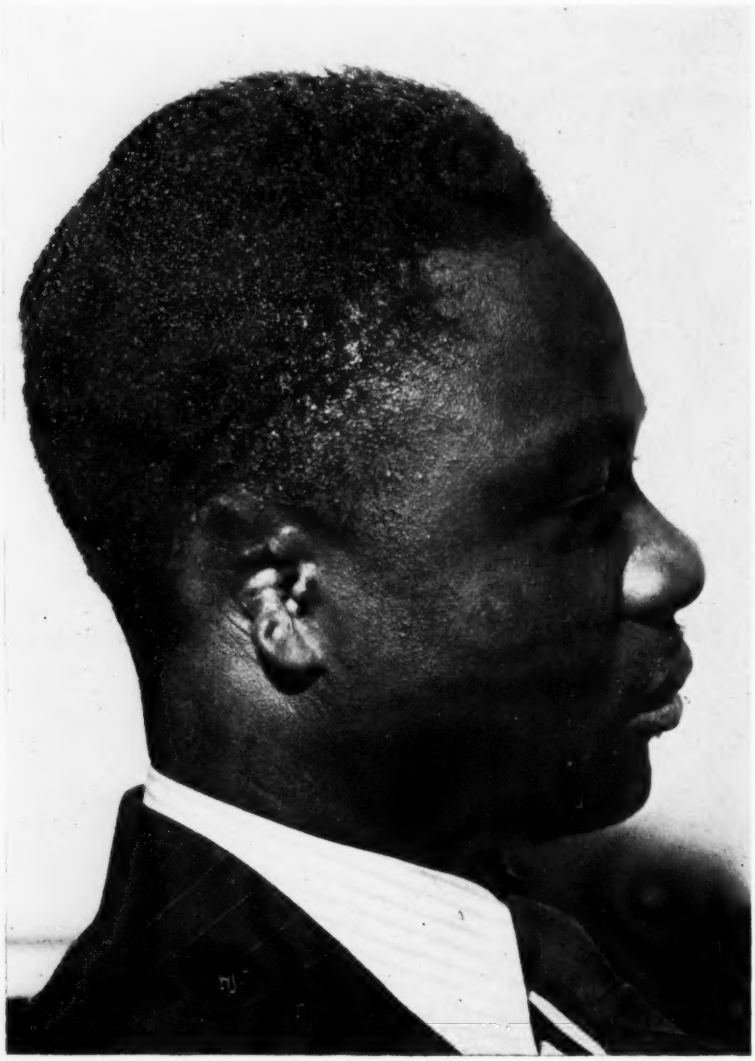

Fig. 5.—A side view of a patient requiring a prosthetic ear.

Oil-soluble dyes serve their most important purpose in obtaining pink-blended flushlike effects on the cheeks, lips, tips and ala of the nose, and knuckles, and blue effects of veins that lie beneath the skin.

These results are obtained by preparing a mixture of one part of turpentine and one part of benzine as a medium. Into 50 c.c. of this mixture are dissolved 10 Gm. of the oil-soluble dye. This will produce a heavily concentrated dye mixture which may be considered a stock solution. Before it is

used a drop or two of the solution is added to a few drops of the benzine-turpentine mixture. The diluted mixture is applied with a brush to tint the prosthesis at the desired places. It is advisable to try to blend at the time the color is applied, but it should be realized that the resulting blended effect takes place automatically by bleeding rather than by the artistic skill of the worker. As the colors bleed they become lighter; therefore, the tints are applied more intensely than desired in the finished prosthesis. After applying the oil-soluble dyes, the prosthesis may be left to stand or it may be washed immediately with soap and water to test the permanency of the dyes employed. If the right dyes are used and the work done properly, it will be impossible to wash off the dyes.

The following is a list of oil-soluble dyes suitable for this purpose which are manufactured by Du Pont:

Anthraquinone Blue AB Base
Anthraquinone Blue SKY Base
Anthraquinone Green G Base
Anthraquinone Iris R Base
Oil Red
Oil Black BG
Oil Brown N
Oil Yellow N
Oil Yellow
Oil Fast Yellow EG

4. *Alcohol-Soluble Dyes.*—The alcohol-soluble dyes have their place in prosthetic work, but they are not used to the same extent as those that are oil-soluble. The stock solutions of these dyes are made up in the same manner; that is, 10 Gm. of dye are added to 50 c.c. of alcohol. The intensity of the dye can be reduced as it is used by adding a few drops of dye solution to the alcohol until the desired shade is obtained. The diluted solution is applied to the prosthesis with soft brushes. Blended surface effects can be obtained. These dyes remain where they are placed and do not bleed into the rubber. In some cases it is desirable to put a thin coat of matte lacquer over the finished prosthesis, which in turn makes the alcohol-soluble dyes adhere more readily to the rubber surface. This will be discussed more fully later.

The following alcohol-soluble dyes manufactured by Du Pont are suitable for use on rubber:

Luxol Fast Red BB
Luxol Fast Red B
Luxol Fast Scarlet C

5. *Applying Hair, Artificial Eyes, and Fingernails.*—For the sake of naturalness it is sometimes desirable to imbed hair into the rubber cast. Each hair is usually inserted separately with a needle. The tip of the eye end of a small needle is filed until it is open at the end. Then the pointed end of the needle is fixed into a small piece of wood which serves as a handle. To insert the hair a pointed needle is used to pierce the rubber and the forked end of the prepared needle is used to straddle the end of the hair over the pierced rubber.

By applying pressure to the hair with the forked needle over the hair, it is forced into the previously formed hole. The strength of the rubber closing over the hair is generally sufficient to hold it in place. If the hair is placed into a hollow rubber cast, such as a masculine hand filled with a microcrystalline wax core, however, the wax will give additional adhesion in holding the hair in place.

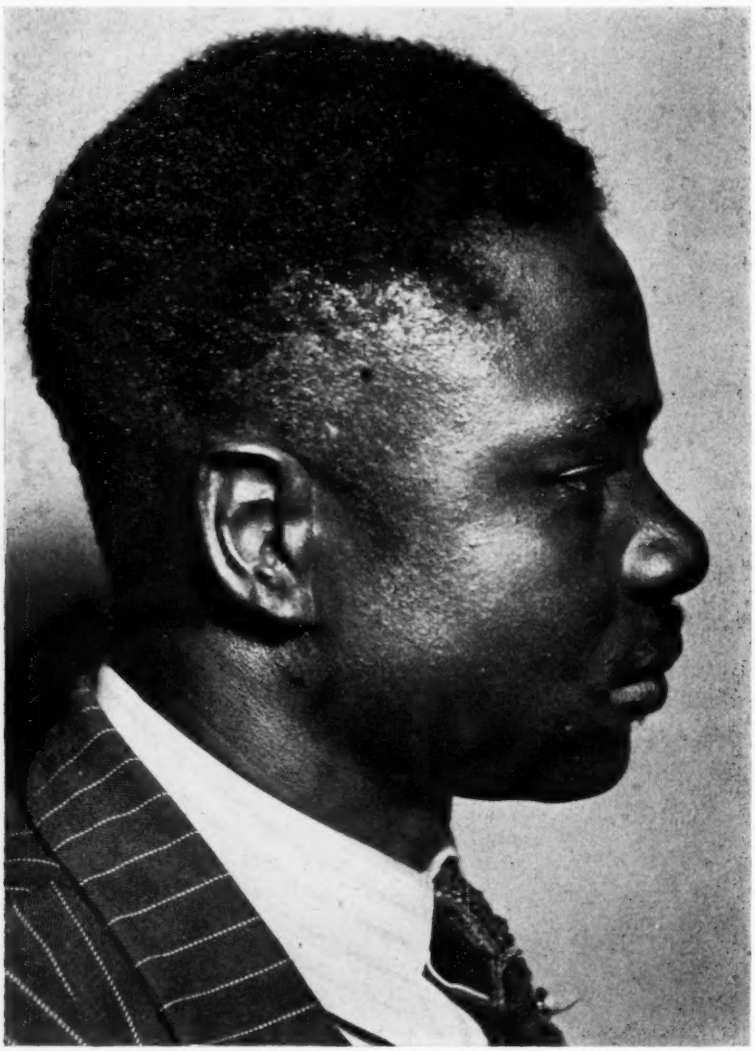

Fig. 6.—The same patient with the ear in place.

A large area of hair as, for example, a mustache, may be applied to a male patient to hide joining lines where a prosthetic nose and lip connect to the face.

The process of setting hairs in a foundation of gauze, lace, or woven stiff silk is known as *ventilating*. The needles employed for the purpose are *ventilating needles*. These have front hooks of varying sizes, ranging from small to

large. Small ones are used to thread one or two hairs and the larger ones for more strands.

Fig. 7.—The front view of the patient without the prosthesis.

If the prosthetist does not wish to attempt this branch of the art, a professional wigmaker can supply artificial mustaches to match natural hair. Indeed, he can make them from the patient's own hair. Eyebrows may be made in the same way. Although this manner of making mustaches and eyebrows is not the most successful, it may serve the purpose. The most naturalistic eyebrows and eyelashes can be obtained from cadavers. These are cut away, tanned, and softened before use. After removal of the brow or lash the fat of the skin is dissolved out in benzine. The skin is then bleached in hydrogen peroxide or a hyperchlorate solution. It should be noted that the

peroxide will also bleach the hair and this will subsequently have to be dyed to the desired shade. The skin is then treated with tannic acid and finally softened in a weak solution of glycerin or a similar agent. Such natural eyebrows and lashes are easily obtainable by the physician and prosthetist having connection with a school of medicine.

Fig. 8.—A front view of the same patient with the ear in place.

Artificial eyelashes which may be purchased from cosmetic stores and costumers and attached to the human eyelids in order to enhance the beauty of the wearer are not suitable for prostheses because they are so long they give an unnatural appearance. If they are trimmed shorter the ends of the hairs are blunt. Natural eyelashes are tapered to pointed ends. The blunt hairs are obvious on the prosthesis.

The gauze or natural eyebrows are attached to the surface of the rubber with the same latex mix that is used to make the prosthesis. The latex mix may be applied directly to the gauze or leatherized skin with a brush, after which the mustache or eyebrow is set in place. A hypodermic syringe with a large needle may also be used to inject additional latex between the hairs on top of the gauze. By using the syringe and needle the latex can be kept from soiling the hairs.

In order to obtain the best adhesion of the latex to the set rubber, the prosthesis should first be wiped off with a cloth dipped in alcohol. This removes grease and foreign matter that may prevent the gauze or leather from sticking. It is also more natural to insert a few stray hairs into the rubber around the mustache or eyebrow.

Artificial eyes may be purchased from the larger optical and taxidermal supply stores. Those furnished by the taxidermist are globe-shaped, whereas those supplied by the optical stores are shell-shaped. The shell-shaped eyes are more expensive but better to use. The globe-shaped eyes, however, may be cut to size with a three-cornered file. In any event, the artificial eye is first matched to the real eye of the patient and fitted into the wax pattern. The wax is fitted to the patient to be sure the eye will look natural. A plaster mold is made over the eye and wax pattern. Before the rubber latex is poured into the mold the eye must be removed from the wax and replaced by itself into the mold. It may be held temporarily in its original place by a small piece of sticky wax. The mold is then closed and the latex mix poured. After the set rubber is removed, it may be necessary to trim some of the rubber where it joins the eye. This, of course, is done with hot tools. If the patient has one good eye it is logical that the glass eye incased in a rubber prosthesis will not move with the natural eye. Unfortunately, this cannot be helped but it will be less noticeable if the patient wears eyeglasses with one dark lens and one clear lens, or with two slightly tinted lenses.

If a prosthetic hand is well done it is seldom necessary to use artificial fingernails, especially with hands of men. It is advisable, however, to apply natural cellulose acetate fingernail lacquer to the rubber nails. The average woman prefers artificial nails which may be purchased from cosmeticians and five-and-ten cent stores. They are applied by simply trimming them to fit the particular rubber fingers and then sealed in place with cellulose acetate or polyvynl acetate cement. Some natural fingernail lacquers also may be used for affixing the artificial nail to the rubber fingers.

6. *Highlighting or Lacquering the Surface.*—It is not always necessary to lacquer the surface of a rubber prosthesis, but frequently this is desirable, especially to cover lips and eyelids. The lacquer on rubber often makes the rubber more receptive to cosmetics; because the rubber surface causes a "drag" on the fingers and brushes that are used in applying make-up. The wearer of a prosthesis must learn to use the lacquer as well as cosmetics. If he gets a better effect with lacquer he must clean the cosmetics and old lacquer from the prosthesis with alcohol after the prosthesis is removed. He then applies an extremely thin coat of matte lacquer before retiring for the night. The next morning the lacquer is thoroughly dry and the prosthesis is

ready for reapplying and is receptive to the make-up. Since the lacquer is alcohol-soluble, the adhesive for holding the prosthesis in place, which is also alcohol-soluble, must be applied with care to the reverse side to prevent mixing and the formation of a gum deposit on the edges. Although the lacquer is not essential and is seldom used by masculine patients, it has its place in creating special effects. It should also be realized that lacquer will eventually wash from the surface; it should not be considered as a permanent part of the prosthesis. If the prosthesis lacks translucency a matte lacquer will create an effect of translucency. A primary use of lacquer is to set or hold any surface coloring with alcohol-soluble dyes and to make the surface more receptive to the application of cosmetics, as previously mentioned. It is well known that many face creams contain an oil or grease in one form or another. These oils and greases readily attack and deteriorate rubber if used in excess. The prosthetist can use cosmetics that have no oil or small amounts of oil or grease in them, or he can make the surface of the prosthesis impervious to the action of cosmetics. Face creams and lotions containing stearic acid may be used as a powder base, because the stearic acid does not readily attack the rubber. Therefore, vanishing creams may be used, whereas cold creams should be omitted. This subject will be discussed more fully under *Cosmetics.*

If lacquer is used it should be alcohol-soluble and kept quite thin. Most lacquers can be diluted with acetone; others may be diluted with alcohol to acquire this thin consistency. This applies particularly to the polyvinyl acetate lacquers. A formula most suitable for this purpose is as follows:

	Parts by weight
Ethyl alcohol	700 Gm.
Ethylene dichloride	200 Gm.
Amyl acetate	80 Gm.
Castor oil	20 Gm.
Polyvynl acetate	100 Gm.

The liquids are mixed together and the polyvynl acetate crystals are added. The container is shaken from time to time until the polyvynl acetate is dissolved completely.

Commercial lacquers are invariably too brittle on drying for application to rubber, which undergoes considerable bending and stretching. It may also be necessary to add a small amount of castor oil to the commercial lacquer to make it more plastic on drying. If the lacquer cracks or develops other defects that make it undesirable, it may be removed from the prosthesis with a cloth dampened with alcohol. Lacquer that is not alcohol-soluble may be removed with a cloth dampened with acetone or amyl acetate.

7. *Cosmetics.*—As in the case of lacquers, cosmetics are not absolutely essential to a well-prepared and colored prosthesis. Because of the darkening effect of sunlight on rubber and the changing of skin tints as a result of the various seasons, however, cosmetics may be employed to counteract these gradual differences. Furthermore, cosmetic pastes are used to fill *join lines* to make them invisible. *Join lines* result where the prosthesis meets the natural

skin. In any event, cosmetics should be used sparingly. Any semblance to stage make-up should definitely be avoided. Opaque pastes and lotions should not be used, or the translucency of the rubber and skin will be lost and produce an unnatural appearance. The following general remarks on the application of prostheses and make-up to the face should be studied carefully. It should be remembered that there are a variety of facial prostheses, such as eye sockets, noses, ears, and depressions or scar casts.

1. The face is washed with a mild soap. If the patient has a tendency toward oily skin, an astringent should be used after washing. Practically any astringent is suitable for this purpose. A satisfactory one may be prepared as follows:

Alcohol	1,000 c.c.
Menthol	4 Gm.
Glycerin	50 c.c.
Water	1,300 c.c.
Perfume	q.s.
Color (vegetable dye)	q.s.

The menthol is dissolved in the alcohol and the other ingredients are added in the order mentioned.

2. The adhesive is placed on the side of the prosthesis that comes in contact with the skin surfaces. It should not extend to the front or back beyond the area that is made to fit the face. I have found the following formula suitable for this purpose:

Gum mastic or colophony	175 Gm.
Ether	200 c.c.
Alcohol	200 c.c.
Castor oil	10 c.c.

The gum mastic is placed in a bottle; the castor oil is mixed with the alcohol and then added to the gum mastic. Finally, the ether is poured in and the bottle is shaken well. In a day or so the gum mastic will be dissolved completely. Impurities in the mastic, such as dirt or pieces of bark, settle to the bottom. The clear liquid is poured off and put into smaller bottles to be supplied to the patient. The gum mastic in this formula is the adhesive agent. The ether is for quick drying and the alcohol is for slower drying. Some patients prefer to use ether as the solvent for gum mastic, because they can put the prosthesis in place the moment the adhesive is applied. Others prefer to use alcohol only, since it gives them a minute or so to wait before putting the prosthesis in place. The castor oil is used to prevent the mastic from crystalizing on drying. Colophony (rosin) can be substituted for the gum mastic. I know of two cases that developed a rash from the use of gum mastic. In such a case another gum should be substituted.

3. After the prosthesis is in place a pastelike putty should be applied over the join line where the prosthesis comes in contact with the human skin. This is particularly necessary for noses and eye sockets, but is not essential in prosthetic hands. The putty is used sparingly on small artificial portions of

the ear to cover the join line where the lobe or top curvature meets the natural ear. If an entire ear is made the join line can be planned at the least conspicuous place next to the head. A join line in the front of the ear should be made invisible.

A theatrical nose putty can be purchased from drug stores. This material is difficult to use, however. A far more satisfactory product can be prepared as follows:

Be Square special wax, 160 to 165 m.p., amber (Bereco Oil Co., Tulsa, Okla.)	20 Gm.
Mineral oil	5 c.c.
Rosin (water white)	20 Gm.
Talc	20 Gm.
Red color (oil-soluble)	q.s.
Perfume (oil or alcohol-soluble)	q.s.

The mineral oil and wax are melted together; the rosin is then added and the mixture stirred over a low fire until completely melted. It is removed from the fire and the talc added. The mixture is stirred until cold. If too sticky more talc is added while cold, and the mixture is kneaded like dough until it will not stick to the fingers. A drop of diluted oil-soluble red color and a drop of perfume are added and the kneading is continued until the correct color is obtained. The wax gives plasticity to the composition, and the rosin causes adhesiveness. The mineral oil is used to overcome the dryness produced by the talc and to improve the pliability. The talc counteracts the extreme stickiness of the mixture and reduces the excess translucency. This putty should be of a consistency that bends readily without cracking as the face assumes different expressions. For winter use, a few additional drops of mineral oil may be added. The color should be of a shade and quantity to suit the individual. This is of no great consequence, however, since a stock supply can be made up without the color. The color can be added to a small amount of the stock supply while kneading the putty in the fingers. From time to time the putty is held next to the patient's skin for matching. When the correct tint is reached the putty is given to the patient for use.

This mixture is employed to cover join lines in the following manner. The fingers are dipped in water to keep the putty from sticking to them. A small piece is rolled between the fingertips until it is cylindrical and about the size of a toothpick. This is laid over the join line and pressed into place with a wet finger. It is then smoothed out until the prosthesis blends with the face and the join line disappears.

4. After the join line is covered a skin lotion or vanishing cream may be used as a powder base to cover both the prosthesis and the face. This should not be of an oily nature and should be applied sparingly. A small amount is dappled on the forehead, cheeks, and chin, then blended with the fingertips into the skin and prosthesis until the lotion disappears.

The following formulas devised by the Atlas Powder Company are most satisfactory for use with prostheses for powder bases, vanishing cream, skin lotion, and similar preparations:

	Ingredients	Formulas 1	2	3	4
A.	1. Arlex	3.0	8.0	5.0	2.5
	2. Potassium carbonate	0.7		0.7	
	3. Potassium hydroxide		1.0		
	4. Triethanolamine				1.7
	5. Water	76.4	64.1	45.1	70.9
	6. Preservative	q.s.	q.s.	q.s.	q.s.
	7. Stearic acid	15.0	20.0	10.0	3.5
	8 Lanolin	0.5	1.5	0.5	2.0
	9. Mineral oil	2.0	2.0	35.3	15.0
B.	10. Mannitan Monooleate (Atlas G-954)	2.0	1.0	1.0	2.0
	11. Mannitan Monostearate (Atlas G-908)			2.0	2.0
	12. Spermaceti		2.0		
C.	13. Perfume	0.4	0.4	0.4	0.4

METHOD OF MANUFACTURE

Warm A and B separately to 75 to 80° C.

Add B and A slowly and with thorough agitation.

Add C at 50° C.

Note: Stir No. 1 and No. 2 intermittently, and when body has increased, turn over by hand. No. 1 cream may be poured directly into jars at 58° C.

No. 4 cream must be passed through colloid mill or homogenizer while still fluid.

No. 3 cream should be stirred until cold, set aside over night, and remixed the following morning.

No. 1, Vanishing Cream
No. 2, Hand Cream
No. 3, All Purpose Cream
No. 4, Liquid Cream

MODIFICATIONS

For modification of the above formulas the following will serve as guides:

Foundation Cream.—Formula No. 2. Add 2 per cent titanium dioxide.

Powder Cream.—Formula No. 2. Add 5 per cent titanium dioxide and color lakes.

Emollient Skin Cream.—Formula No. 3. Increase lanolin to 5 per cent or as required.

Liquid Cleanser.—Formula No. 4.

Hand Lotion.—Formula No. 4. Reduce mineral oil to not over 5 per cent, increasing water by like percentage.

Liquid Powder Base.—Formula No. 4. Reduce mineral oil to 5 per cent as above.

5. After the powder base is applied and dried, moist rouge or powder cake rouge may be blended into both the skin and prosthesis at the proper places. The moist or cream rouge serves best for this purpose because the dry cake rouge does not spread evenly over rough or hairy skin and enlarged pores Some rouges have a tendency to permanently dye the rubber. If the rouge

used cannot be removed from the prosthesis with alcohol it should not be considered for prosthetic work. Rouge also can be used to good advantage on the ala of a prosthetic nose or the top curvature and lobe of a prosthetic ear if the prosthesis lacks color at these points or if fugitive colors were used in making the prosthesis. The rouge should be applied sparingly and blended with the fingertips. In making up a prosthesis the tendency should always be toward naturalness without in any way leaning toward the theatrical. The main object is to prevent a "made-up" appearance and to avoid attracting undue attention to the face.

6. Any face powder of suitable shade and texture can be used over a proper powder base. It should be dusted on lightly and blended over the whole with a soft powder puff. It, too, should be applied sparingly to prevent an opaque cast to both natural and artificial skin. Powder applied to the skin has a tendency to kill the natural sheen and high lights. Often it is desirable to restore the natural qualities of the skin. A cold moist towel gently pressed against the face and prosthesis will set the powder and at the same time restore natural skin characteristics. This is especially desirable for a male patient. The powder aids greatly in hiding properly filled join lines.

7. Lipsticks or paste can be used in the usual manner over both rubber and natural lips, but should not be used in excess, since the grease content may cause the rubber to deteriorate unless it is lacquered. Indelible lipsticks should be avoided because they stain the rubber to such an extent that the color cannot be removed. It is good practice to test a lipstick on the back of a prosthesis to see if it can be removed properly before using it on the lips.

8. Eyebrow pencils can be used if desired on rubber prosthesis with hair. Such prostheses should be made to withstand washing with mild soap and water and an occasional cleaning and disinfecting with alcohol.

9. Brilliantine and similar hair preparations can be applied with a tooth or mascara brush on the hair of a prosthesis. Such mixtures should be used with care and very sparingly, however. An excess of oil in the artificial hair collects dust and foreign particles. This necessitates frequent cleaning, resulting in premature deterioration of the artificial hair, and replacement becomes necessary.

Editorial Announcement

Previously it has been customary to end the volumes of THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE with the September issue. A change has been made, however, by which the future volumes will conform to the calendar year, beginning with the January issue and ending with that of December. The present volume 28 will, therefore, continue through the December number, and the index which has been omitted from this number will appear instead in that issue.

Editor.

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